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The Scientific Meeting, consisting of ten symposia, sixteen workshops, three poster sessions, a presidential address, a keynote address, an award lecture and a student award session was held in Dublin, Ireland, September 20-24, 2003. The scientific program Committee consisted of Luke O'Neill, chair, Andrew Bowie, Fionnuala Brennan, Charles Dinarello, Brian Foxwell, Andy Gearing, Pietro Ghezzi, Jim Johnston, Dermot Kelleher, Kingston Mills, Paul Moynagh, Anne O'Garra, Joe Oppenheim, Jeremy Saklatvala, John Sims, and Clair Gardiner. Progress in the vital area of cytokines was described by major investigators in both basic science and clinical applications. Detailed summaries of these advances are discussed in the attached proceedings of the meeting. It was a very successful meeting.

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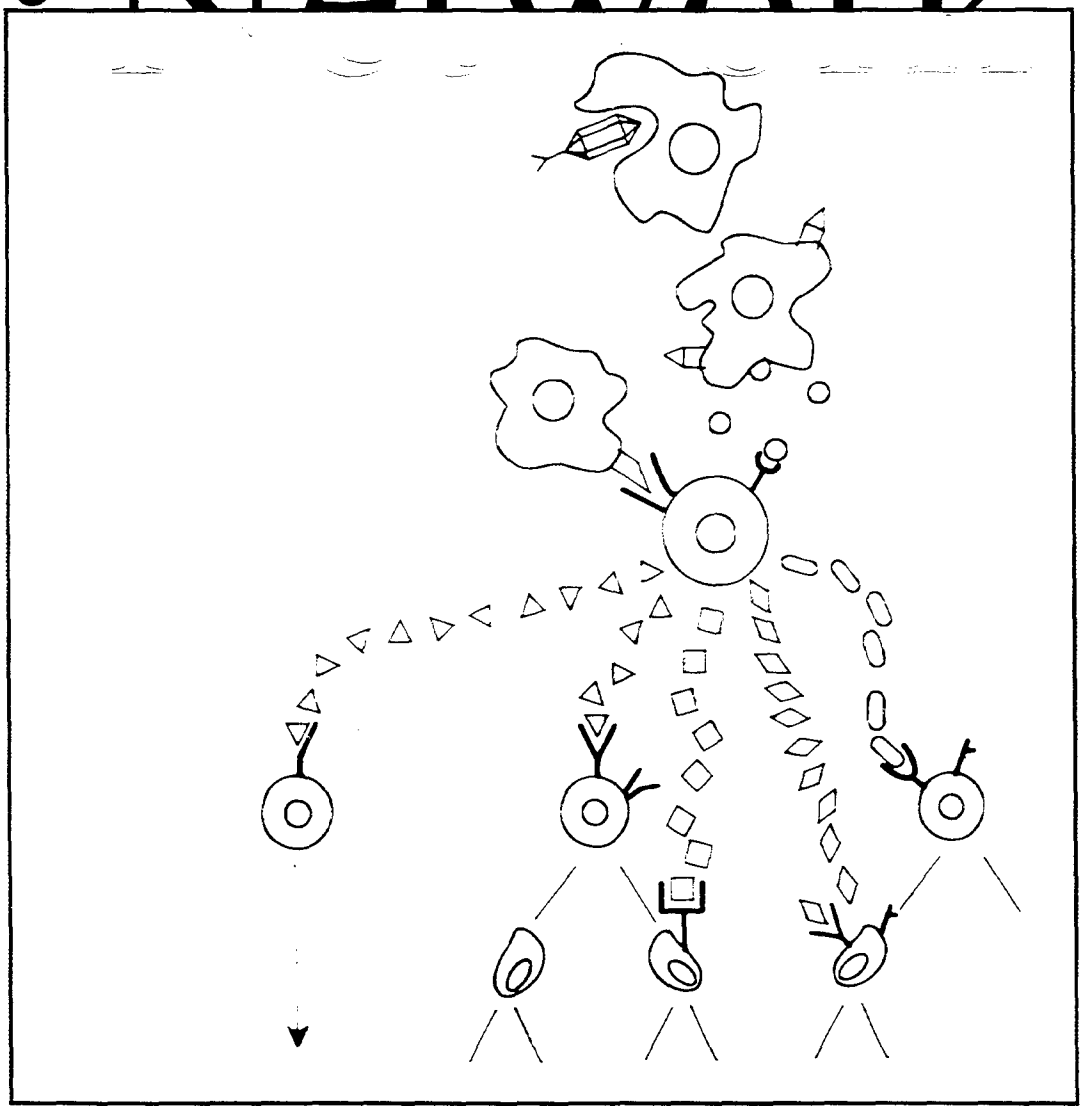

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The Eleventh International Cytokine Conference
Dublin, Ireland
September 20-24, 2003

Proceedings/Report
Massimo Gadina and Antonio Sica

The meeting report has been prepared by Massimo Gadina (Belfast) and Antonio Sica (Milan) and, as usual, is only a partial coverage of the meeting

Signal transduction
The opening lecture was given by **Inder Verma** (The Salk Institute, La Jolla, USA), who presented new data on the role of IKK1 and IKK2 in development. While NF-κB activation is not completely abrogated in the absence of IKK2, mice deficient for IKK2 have tremendous apoptosis in the liver and die. IKK1 -/- deficient mice die at day 0 and have important impairment of cranial/faces bone formation. Additionally, IKK1/IKK2 double KO do not show complete formation of the brain (additional phenotype) and lack NF-κB activity. Blockiage of NF-κB results in mesoderm malformation (major role in development).

Jules Hoffmann presented a broad overview of the Drosophila antimicrobial defence. Elucidating the pathways that regultaes the production of two of this peptides Drosomycins andf Diptericins is regulated by two independent pathways. The *Toll* pathway, which is critical for Drosophila antifungal response and for Gram-positive bacterial infections, is responsible for Drosomicin production whereas Diptericins production is under the control of the *Imd* pathway, the homologue of the TNF pathway in higher species.
Rob Kastelein from DNAX reviewed the IL-12 family of cytokines focusing in particular to the two new members named IL-23 and IL-27. IL-23 appears to be more important for memory T cells. In vivo this is underscored by the fact that IL-23 deficient mice have normal Th1 development but are resistant to autoimmune diseases such as Experimental Autoimmune Encephalitis and Collagen Induced Arthritis. IL-27 binds to a receptor composed by WSX-1/TCCR and gp130 which is also utilized by a wide variety of cytokines. Biologically, IL-27 appears to be important in controlling resistance to parasites such as *Toxoplasma gondii*.

The role of inhibitory molecules in cytokine signalling has been addressed by several speakers. **Jim Johnston** from Queen's University in Belfast presented some interesting data which link the expression of SOCS3 to allergy and atopy. From studies performed in animal models and

subsequently confirmed in patients with such pathologies it appears that SOCS3 is preferentially expressed in Th2 cells and can potentially utilized as a marker to measure the severity of the allergic disease. **Doug Hilton** also presented data from various animal models. In an impressive effort to shed light on the physiological role of SOCS molecule, his group at Walter and Eliza Hall Institute in Melbourne has generated knockout mice for all the known SOCS. He is now directing his attention to a series of mutant mice generated by ENU mutagenesis to identify new molecules important for the immune response. Similar approach has already been undertaken by several group including **Bruce Beutler** who has successfully utilized this approach to identify two genes LPS2 and dsRNA1 who are involved in the innate immune response. The SOCS molecules have been at the centre of **Aki Yoshimura**'s presentation. In an elegant study aimed to define the effects of SOCS1-deficiency in DCs in vivo, his group generated mice in which the SOCS1 expression was restored in T and B cells on a SOCS1-/- background. These mice, showed thymic and splenic accumulation of DCs and an abnormal expansion of B cells with increased autoantibody production.

Ken Murphy (Washington University) recent work focused on a new lymphocyte attenuator, BTLA engagement results in down regulation of T-cell activation, and mice deficient in BTLA show increased incidence and severity of autoimmune disorders.

John O'Shea (NIH) has collaborated with Paul Changelian at Pfizer in the generation and testing of a new compound designed to block the tyrosine kinase activity of Jak3. The potency and the selectivity of such molecule appears to be adequate for possible use in a clinical setting.

Toll-like receptors

The Cytokine Conference in Dublin was characterized by a great emphasis on the TLR system, a signature of Luke O'Neill (somebody mentioned that this was a TLR meeting disguised as a cytokine meeting). A number of outstanding reports presented novel findings in this field and focused the attention on the role of different adaptor molecules in TLRs-mediated gene transcription. Beside MYD88, several novel adaptor molecules including TIRAP/Mal and TRIF have recently been identified and discussed at the meeting. **Shizuo Akira** (University of Osaka, Japan) has provided a substantial amount of information on the differential utilization of these adapter molecules by TLRs signaling. Both TLR4 and TLR3 have MYD88 independent pathways which promote the expression of IFN-inducible genes (IP-10, GARG16, IRG1) via IRF3. Activation of the MYD88-dependent pathway results in TRAF6 engagement and the expression of inflammatory genes (IL-12 and TNF). TRIF (TIR containing adaptor inducing IFN-? or TIR-containing adaptor molecule-1 (TICAM-1) possesses the ability to activate not only the NF-?B-dependent but also the IFN-? promoters. Deletion of N-terminus acts as a DN inhibitor. TLR3 dependent activation of IFN-? is inhibited by TRIF-DN. PolyIC-induced NF-κB (TLR3) activity is abolished in TRIF-/- fibroblasts. The TRIF-/- fibroblasts also suppressed the LPS-induced

expression of IFN-inducible genes. The N-terminal portion of TRIF associates to TRAF6. in yeast, in its N-terminus. Disruption of the TRAF6-binding motifs of TRIF resulted in a reduction in the TRIF-induced activation of the NF-κB-dependent but not IFN-? promoter. TBK1 (TANK-binding kinase) associates with TRIF and activates the IFN? promoter. TRAF6 and TBK1 associates with TRIF at different regions.TRAM (TRIF-related adaptor molecule) is a TIR domain containing adaptor molecule, which promotes the late phase of activation of NF-κB. TLR4-dependent activation of IFN-inducible genes expression are severely impaired in TRAM -/- mice (no for TLR3). TLR4-dependent late phase of NF-κB and JNK activation is impaired in TRAM-/- mice.

Doug Golenbock (University of Massachuttes, USA) pointed out that LPS/TLR4 signaling to IRF3/7 and NF-?B involves TRAM and TRIF. These two adapter molecules likely cooperate to regulate the MYD88-independent pathway downstream of TLR4 during the innate response to lipopolysaccharide. By using plasmacytoid dendritic cells, he has also shown that once the TLR9 ligand CpGDNA is engulfed by cells and goes to the tubular compartment, MYD88 is recruited to these CpGDNA containing structures. This signaling pathway is entirely dependent on MYD88 and non-stimulatory CpGDNA can compete for stimulatory CpG sequences,

Matthew J. Fenton (University of Maryland, USA) has studied the differential role of TLR4 and TLR2 in macrophage activation. TLR2 agonists induce expression of a more limited repertoire of pro-inflammatory genes than TLR4 agonists. The effects elicited by TLR2 and TLR4 on other pathways (STAT-1, PI3K, PKC, PKR) were also discussed.

A number of presentations discussed the role of Inhibitors of TLRs-mediated responses. **Luke O'Neill** (Trinity College, Dublin) has shown that T1/ST2 inhibits IL-1 and LPS, but not polyI:C-induced NF-?B activation. T1/ST2 is a member of the IL-1 receptor family possessing the characteristic extracellular Ig domain and an intracellular TIR domain. It is involved in the Th2 response and has been shown to inhibit LPS-induced cytokine production. No inhibition was observed on either NF-κB, ISRE in cells stimulate with PolyI:C. Overexpression of T1/ST2 inhibits NF-κB activation by IL-1RacP, TLR4, Mal, MYD88 but not IRAK or the TLR3 adaptor TICAM-1. A GST-fusion of the TIR domain of T1/ST2 is shown to bind to Mal and MYD88 and not to TICAM, indicating that the observed inhibition of IL-1 and LPS may occur through sequestration of the intermediate signalling molecules. T1/TS2 is therefore an inhibitor of IL-1 receptor family with respect to NF-κB, consistent with its role in the Th2 cell regulation.

Xiaoxa Li (Cleveland Clinical Foundation, Cleveland) showed that SIGIRR is a negative regulator of IL-1R-, TLR4- and TLR9-mediated signaling. Inflammation is enhanced in SIGIRR-null mice (enhanced chemokine induction after IL-1 injection) and a reduced threshold for lethal endotoxin challenge. Biochemical analysis indicated that SIGIRR bind to IRAK and TRAF6, through its TIR domain.

Taro Fukao (Centre d'Immunogie Merseille Luminy, France) proposed that PI3K acts as a negative regulation of TLR signaling. PI3K negatively regulates TLRs-induced IL-12 synthesis by DCs. TLRs stimuli inducing IL-12 production concomitantly elicit PI3K activation in DCs, but both PI3K -/- and PI3K inhibitor treated DCs demonstrate increased IL-12 production. Consistently, activation of several signal transduction pathways triggered by TLRs (e.g. p38) are

enhanced in DCs with low PI3K activity. PI3K -/- mice infected with *Leishmania major* have higher Th1 response. Thus, PI3K activation may prevent excessive Th1 polarization.

The Meeting covered important issues in the area of chemokines research. **John O'Shea** (Bethesda, Maryland) provided evidence that the Jak/STAT pathway does not affect chemokine receptor functions. Jak2 and Jak3 -/- does not impair CXCL12 dependent chemotaxis or Ca++ flux. He has also shown that the serine/threonine protein kinase Tpl2/Cot, which is involved in the ERK pathway, is induced by IL-12 and is expressed by Th1 cells. He suggested that Tpl2/Cot is involved in Th1 differentiation.

Ann Richmond (VA Medical Centre, Nashville, USA) reported crucial data about the modulation of CXCR2 after ligand stimulation, presenting information about the internalization, recycling and resensitization of the receptor. The study demonstrates that the carboxyl terminal domain of the CXCR2 plays a major role in the interaction with adaptor proteins, that leads to the internalization of the receptor, a major event in the polarization of intracellular signals. Proteins such as beta-arrestin, AP-2, HIP, and PP2A have been recognized as important elements in the processing of the activated CXCR2 so that after ligand desensitization, the receptor can be internalized, resensitized and recycled back to the membrane or be targeted for degradation in the lysosome. The trafficking of the receptor within the different cell compartments was studied by means of colocalization studies with the main components of the Rab protein family, such as rab5, rab11 and rab7. They also suggested the fact that colocalization of CXCR2 with the C-terminal domain of myosin Vb is a key event in the resensitization and recycling of CXCR2 and in ligand mediated chemotaxis.

Another important talk related to chemokine receptors trafficking was made by **Mark Marsh** (Laboratory for Molecular Cell Biology, University College London, UK) on the trafficking of Cellular and Viral Chemokine Receptors, specifically CCR5 and US28 respectively. Using electron microscopy. Marsh and colleagues have demonstrated that upon ligand binding to CCR5 a fast redistribution (clear alignment) of the receptors in the plasma membrane takes place. This is followed by internalization through endocytic clathrin coated pits and vesicles. The entry to the early sorting endosomes and the passage of the receptor to recycling endosomes where resensitization may occur was discussed. The second part of the talk focused instead in the HCMV derived chemokine receptor US28 and its "preference" for late endosomes. The internalization of this receptor is beta-arrestins independent and clathrin-mediated pathway dependent. This receptor that signals constitutively may be responsible for the continuous clearing of the chemokines surrounding the infected cells.

Martin Lipp (Max Delbruck Centre, Berlin, Germany) discussed the effect of CCR7 and CXCR5 on lymphoid organ development and adaptive immunity. Using knock out mice for CCR7, CXCR5 and their ligands, they have characterized the role of these molecules in the organization of the secondary lymphoid tissues and the efficiency of B and T cell-mediated immune responses. CCR7 and CXCR5 have also been identified as useful markers in the classification of functionally distinct subsets of T helper cells, which may lead to a better understanding of T cell memory and its effector functions.

Ben-Baruch (Tel Aviv University) has found that breast cancer cells produce high level of CCL5 and CCL2, which support the malignant process by inducing monocyte infiltration. CCL5 and CCL2 increase the production of MMP and TNF-α by monocytes, which in turn induces MMP and CCL5/2 secretion by tumor cells. CCL5 and CCL2 mediate a pro-malignant interplay between the tumor cells and Tumor associated macrophages (TAM). TAM-derived TNF-α is a master regulator of CCL2, IL-6 and MMP expression.

T cell regulation

Muriel Moser (Universitè Libre de Bruxelles, Belgium) discussed the role of dendritic cells in the control of immunity and peripheral tolerance. Immature DC induce clonal expansion of CD4+ T cells in vivo but not their differentiation into IFN-? and cytokine producing effector cells. Neutralization with anti-IL-10R mAb restores the IFN-? production. Interaction of immature DCs induce hyporesponsiveness in vivo. Injection of immature DC induces 3 populations of regulatory T cells: Tr1, Th3, CD4+CD25+. Treg cells exert a negative feedback mechanism on Th1-type response induced by mature DC in vivo.

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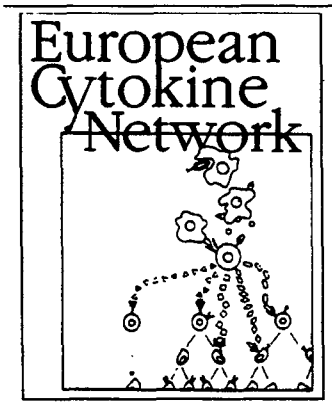

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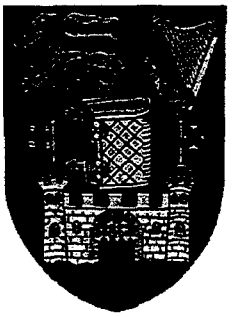

International Cytokine Society Annual Meeting

Trinity College, Dublin, Ireland

September 20th-24th, 2003

Organising Committee

Luke O'Neill (Chair), Andrew Bowie, Fionnuala Brennan, Charles Dinarello, Brian Foxwell, Clair Gardiner, Andy Gearing, Pietro Ghezzi, Jim Johnston, Dermot Kelleher, Kingston Mills, Paul Moynagh, Anne O'Garra, Joe Oppenheim, Jeremy Saklatvala, John Sims.

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Speakers

1

CHEMOKINE MIMICS: EFFECTS OF DEFENSINS AND AUTOANTIGENS ON NORMAL DENDRITIC CELLS

Joost J. Oppenheim,[1] De Yang[1], Arya Biragyn[2], Rachel Caspi[3], Paul Plotz[4], Zack Howard[1]
[1]Lab Molecular Immunoregulation,
[2]Experimental Transplantation Immunol Branch, CCR, NCI,
[3]NEI and [4]Intramural Res, NIAMSD, NIH, Bethesda, MD

Mediators engaged in innate immunity also rapidly amplify adaptive immune responses. For example, epithelial cell derived β defensins chemoattract resting naive T cells and immature dendritic cells (iDC) via CCR6, while LL37 chemoattracts phagocytes and T lymphocytes by interacting with the FPRL-1 receptor. The in vivo relevance of these observations was demonstrated by showing that murine β-defensin-2(muβDF-2) acts as a potent vaccine adjuvant that can enhance immune responses to tumor antigens. Linkage of muβDF-2 to tumor antigens markedly augmented antitumor responses presumably by delivery of the antigen to receptors on antigen presenting mature DC. In addition, muβDF-2 activated DC to mature by interacting with TLR4. We have discovered another set of endogenous molecules, namely autoantigens, can chemoattract T cells and iDC. The myositis auto- antigens histidyl-tRNA synthetase uses CCR5 and asparygyl-tRNA synthetase uses CCR3 to chemo-attractant T cells and iDC, while the uveitis autoantigens IRBP and arrestin (S-antigen) use CXCR3 and CXCR5. Although internalized by DC, these autoantigens do not induce the maturation of iDC. These observations suggest that self antigens which interact with chemokine receptors on iDC, enter a pathway leading to an adaptive immune response that may contribute to the pathogenesis of autoimmune diseases.

2

NALPS, A PROTEIN FAMILY INVOLVED IN THE ACTIVATION OF PROINFLAMMATORY CASPASES, ARE MUTATED IN AUTO-INFLAMMATORY DISEASES

Jurg Tschopp, Fabio Martinon and Michael McDermott, Institute of Biochemistry, 1066 Epalinges Switzerland and Royal hospital, London

Generation of Interleukin (IL)-1β via cleavage of its pro-form requires the activity of caspase-1 (and caspase-11 in mice), but the mechanism involved in the activation of the pro-inflammatory caspases remains elusive. A newly discovered family of cytoplasmic proteins – the NALPs – has been implicated in the activation of caspase-1 by the Toll-like receptors (TLRs) during the response to microbial infection. Like the structurally related Apaf-1, which is responsible for the activation of caspase-9, the NALP1 protein forms a large, signal-induced multiprotein complex, the inflammasome, resulting in the activation of pro-inflammatory caspases. The inflammasome comprises caspase-1, caspase-5, Asc and NALP1. Expression of a dominant-negative form of Asc in THP-1 cells blocks proIL-1β maturation and activation of inflammatory caspases induced by LPS *in vivo*. Thus the inflammasome constitutes an important arm of the innate immunity.
There is *in vivo* evidence for a crucial role of NALP family members in inflammation. Patients with hereditary fever syndromes and chronic inflammatory diseases carrying mutations in NALP3 have recently been identified.

3

STRATEGIES TO INHIBIT IL-1β AND IL-18 BY VACCINIA VIRUS

Geoffrey L. Smith, Department of Virology, Faculty of Medicine, Imperial College London, St. Mary's Campus, Norfolk Place, London W2 1PG

Vaccinia virus is the smallpox vaccine and the most intensively studied poxvirus. Like other orthopoxviruses it has a large double stranded DNA genome, a cytoplasmic site of replication and about 200 genes. Approximately half of these genes are non-essential for virus replication in cell culture but influence the outcome of infection in vivo. Several of these nonessential genes interfere with the host response to infection by binding cytokines, chemokines or interferons or blocking apoptosis, cellular signalling pathways or the interferon-induced antiviral proteins.
The complexity of these immunomodulatory proteins is illustrated by the strategies vaccinia virus uses to block the production or action of IL-1β and IL-18. In each case vaccinia virus interfers at at least three stages. For IL-1β, vaccinia virus inhibits the cleavage of pro-IL-1β to IL-1β by expressing an intracellular inhibitor of caspase 1. Vaccinia prevents soluble IL-1β reaching IL-1 receptors on cells by expression of a soluble high affinity receptor for IL-1β, and within infected cells it expresses a protein that blocks signalling via the IL-1 receptor by mimicing the TIR domain of the toll and interleukin-1 receptor family. Vaccinia virus displays a similar strategy for inhibition of IL-18. The intracellular inhibitors of caspase 1 and toll-like receptor signalling block IL-18 formation and signalling. In addition, vaccinia virus expresses a soluble IL-18 binding protein that blocks IL-18 reaching it receptors. The role of these immunomodulators in the virus life cycle will be discussed.

4

EXPLORING THE FUNCTION OF IL-1 AND IL-1R FAMILY MEMBERS

John Sims, Amgen, 51 University Street, Seattle WA USA

The IL-1 and IL-1R families contain a number of proteins whose function is obscure. Given the evolutionary relationship of these molecules to their IL-1 and IL-18 cousins, it might be expected that the as-yet uncharacterized family members would mediate responses related to inflammation and innate and adaptive immunity. We have documented that IL-1F6, IL-1F8 and IL-1F9 are capable of activating the signaling pathway in a variety of cell types that leads to the activation of NFκB and MAP kinases. The signals appear to be mediated via the IL-1R family members AcP and rp2. The orphan IL-1R family member SIGIRR suppresses pathway signaling that leads to NFκB activation in response to IL-1F6, F8 and F9 as well as in response to IL-1 and IL-18. Finally, we have identified an alternatively spliced form of the IL-1 Receptor Accessory Protein in which the C-terminal exon in "classical" AcP is replaced by an alternative exon, which appears by sequence homology to reconstitute a TIR domain but which does not allow activation of NFκB in response to IL-1. Expression of the new splice isoform, called AcPb, is restricted to the CNS.

5

POST-TRANSCRIPTIONAL CONTROL OF INFLAMMATORY RESPONSE GENE EXPRESSION

J. Saklatvala, J.L.E. Dean and A.R. Clark, Kennedy Institute of Rheumatology, Faculty of Medicine, Imperial College London, London UK

The inflammatory response has evolved as a rapid defence mechanism against microbial invasion, as well as to limit and repair tissue damage. It involves coordinated expression of many genes whose function is to cause leucocytes to move from the vascular compartment to the site of injury, to activate both leucocytes and resident cells, and to initiate repair processes. Expression of the cytokines, chemokines, enzymes and adhesion molecules involved is controlled at transcriptional and post-transcriptional levels. An important point of regulation is mRNA stability, control of which enables both augmentation and rapid adjustment of mRNA levels. It also provides a means of removing mRNAs whose protein products are no longer required.

The stabilisation of mRNAs of inflammatory response proteins is mediated by the p38 mitogen activated protein kinase (MAPK) pathway. Many of the mRNAs are intrinsically unstable by virtue of AU-rich elements in the 3' untranslated region. Much effort is being directed at defining proteins which bind to these since they may target mRNAs for destruction, and provide the basis of the specificity by which the p38 pathway stabilises mRNAs.

The AU-rich elements direct both rapid de-adenylation of the mRNAs and their further degradation. Activation of the p38 pathway greatly slows the de-adenylation, while having little effect on the decay of the mRNA body.

6

CYTOKINES AND BRAIN INJURY

Nancy Rothwell, University of Manchester, Manchester M13 9PT UK

The proinflammatory cytokines IL-1 and TNFα contribute to ischaemic, excitotoxic and traumatic brain injury in experimental animals, while IL-6, IL-10 and several growth factors appear to exert neuroprotective actions. IL-1ra markedly and reproducibly inhibits diverse forms of experimental brain injury, is an endogenous inhibitor of brain injury, and is currently in early clinical trials in stroke.

The mechanisms underlying cytokine action in neuronal injury have not been fully elucidated. IL-1 and TNFα can induce release of a number of neurotoxins from glia and endothelial cells, promote adhesions and immune cell invasion, and also have physiological effects (eg fever), which may contribute to or exacerbate brain injury. IL-1 also markedly enhances seizure activity in rodents possibly through disinhibitory actions.

The contribution of brain inflammation and specific cytokines to chronic neurodegeneration and to repair and recovery appears complex. TNFα appears to be beneficial in repair and recovery which a number of cytokines have been implicated in Alzheimer's, Parkinson's, ALS, TSEs and multiple sclerosis.

Reference

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7

MODULATION OF CXCR2 INTERNALIZATION, RECYCLING AND RESENSITIZATION EFFECTS POLARIZATION OF INTRACELLULAR SIGNALLING AND LIGAND MEDIATED CHEMOTAXIS

Ann Richmond, Guo-Huang Fan, James R. Goldenring, Lynne A. Lapierre, and Jiqing Sai, VA Medical Center and Vanderbilt University School of Medicine, Nashville, TN.USA

Agonist-stimulated recruitment of adaptor proteins to chemokine receptors, followed by receptor internalization plays an important role in translating the cell's response to an external chemokine gradient. Specifically, the binding of adaptor proteins to the carboxyl terminal domain of the CXCR2 followed by receptor internalization appears to be required for polarization of intracellular signals leading to chemotaxis. When the receptor is mutated so that the appropriate adaptor proteins do not bind in response to agonist, PIP3 does not concentrate along the leading edge of the cell responding to a chemokine gradient. Moreover, FRET analysis reveals failure to polarize the activated rac or CDC42 appropriately in cells responding to chemokine gradient. Similarly, the polarized co-localization of actin cytoskeleton with phosphor-AKT is disturbed. A number of adaptor proteins are recruited to the carboxyl terminal domain of CXCR2 to facilitate the polarization of intracellular signals. We have shown that interaction with beta-arrestin, AP-2, HIP, and PP2A are important in the processing of the activated CXCR2 so that after ligand desensitization, the receptor can be internalized, resensitized, and recycled back to the membrane, or be targeted for degradation in the lysosome. Here we show that the co-localization of CXCR2 with the C-terminal domain of myosin Vb is a key event in the resensitization and recycling of CXCR2 and in ligand mediated chemotaxis. Over-expression of the myosin Vb tail but not wild-type myosin Vb reduces receptor recycling and CXCR2 mediated chemotaxis. These data point to the complex series of protein protein interactions required for a continuous chemotactic response to a chemokine gradient.

8

RNA ELEMENTS THAT ACTIVATE PKR RENDER SPLICING OF INFLAMMATORY CYTOKINE mRNA HIGHLY EFFICIENT

Sarah Namer, Anat Hasson, Farhat Osman and Raymond Kaempfer, Department of Molecular Virology, The Hebrew University-Hadassah Medical School, Jerusalem, Israel

The kinase PKR depends for its activation strictly on dsRNA, a hallmark of virus infection. Once activated, PKR inhibits translation by phosphorylating eIF2α. We discovered a novel role for cellular PKR-activating RNA elements in regulation of mRNA splicing. The *cis*-acting TNF-α 3'-UTR element, 2-APRE, renders mRNA splicing totally dependent on PKR activation and sensitive to PKR inhibitors, e.g., 2-aminopurine or dominant-negative PKR. The 104-nt 2-APRE is located well upstream of the ARE and regulates splicing even when placed inside an intron such that it will be excised. When pre-mRNA contains the 2-APRE, splicing is enhanced greatly by increased expression of PKR. Induction of PKR by TNF-α or other signals during an inflammatory response thus creates a positive feedback loop for TNF-α mRNA splicing. Indeed, TNF-α mRNA is expressed swiftly upon immune stimulation whereas TNF-β mRNA, lacking the 2-APRE, is spliced sluggishly and expressed late. However, insertion of this element into the TNF-β gene rendered splicing dependent on PKR and tenfold more efficient.

IFN-γ mRNA potently downregulates its own translation by locally activating PKR through a pseudoknot in the 5'-UTR (Cell 108, 221-232, 2002). Could activation of PKR by the pseudoknot also regulate mRNA splicing? To examine this point, we created a chimeric TNF-β gene carrying the IFN-γ pseudoknot in its 3'-UTR. The pseudoknot (but not a mutant form unable to activate PKR) rendered TNF-β mRNA splicing both dependent on PKR activation and tenfold more efficient. Located within the 3'-UTR, the IFN-γ pseudoknot did not inhibit TNF-β mRNA translation and instead promoted high-level expression of TNF-β protein. Our results suggest that any RNA structure that is capable of activating PKR can strongly enhance mRNA splicing in dependence on PKR.

9

DENDRITIC CELLS AS INDUCERS AND CONTROLLERS OF IMMUNITY

Muriel Moser, Institut de Biologie et Médecine Moléculaires, Université Libre de Bruxelles, Belgium

Dendritic cells (DCs) display some unusual properties aimed at *sensitizing T lymphocytes* specific for dangerous antigens detected earlier in the periphery. The process of DC maturation is begun by exposure to inflammatory mediators, which convert the primarily antigen-capture mode of the peripheral DCs into a mode specialized for T cell activation. They then migrate into the primary lymphoid organs, where they initiate activation of those T lymphocytes specific for dangerous antigens. Several membrane-bound receptor-ligand pairs are involved in T cell priming, in addition to soluble cytokines.
It is noteworthy that DCs are more than a simple "on/off" switch of the immune response but rather contribute significant polarizing influences on T helper cell differentiation. Various mechanisms have been described by which DCs may regulate the Th1/Th2 balance *in vivo* and include the nature of DC subset, the antigen dose, the recognition of pathogen-derived products by specific receptors such as Toll-like receptors and the cytokines present in the microenvironment. The "plastic" properties of DCs allow them to modulate their function according to the nature of the tissue and the infection and to provide this "decoded" information to T cells.
Recent evidence suggests that, in addition to their well known stimulatory properties, DCs may play a major role in *peripheral tolerance*. It is still unclear whether a distinct subtype or activation status of DC exists which promotes the differentiation of suppressor rather than effector T cells from naive precursors. The role of immature versus mature DCs was investigated *in vivo*. Our data show that both DC types induce the proliferation of antigen-specific $CD4^+$ T lymphocytes *in vivo* and that mature, but not immature, DCs provoke their differentiation into cytokine-producing effector cells.
We next tested whether the naturally occurring $CD4^+CD25^+$ regulatory T (Treg) cells may control immune responses induced by DCs *in vivo*. We characterized the immune response induced by adoptive transfer of antigen-pulsed mature DCs into mice depleted or not of $CD25^+$ cells. We found that the development of MHC class I and class II-restricted IFN-γ-producing cells was strongly enhanced in the absence of Treg. By contrast, Th2 priming was downregulated in the same conditions. We conclude that Treg naturally exert a negative feedback mechanism on Th1-type responses induced by mature DCs *in vivo*.
Collectively, these data show that cells of the dendritic family may not only control immunity but also maintain tolerance to self antigens, two complementary functions that would ensure the integrity of the organism in an environment full of pathogens. As they express a variety of receptors which specifically recognize microbial products, DCs are able to discriminate between self and non self and may therefore enable the immune system to mount potent effector activity to pathogens while silencing self-reactive lymphocytes.

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EDAR SIGNALING IN ECTODERMAL ORGANOGENESIS

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Organs developing as ectodermal appendages (such as teeth, hairs, mammary glands) share similar molecular mechanisms and early morphogenesis. Edar and its ligand ectodysplasin-A1 (Eda-A1) are recently identified members of the tumor necrosis factor receptor (TNFR) and TNF superfamily, respectively. Signaling by Edar activates the NF-κB pathway through the specific adapter protein Edaradd and is required for normal development of various ectodermal organs. Mutations in Edar or other molecules of the same signaling pathway cause ectodermal dysplasias characterized by the absence or abnormal shape of teeth, hairs, and numerous glands such as sweat glands. Studies with mice either lacking the functional proteins of the Edar signaling pathway or overexpressing either the ligand or receptor suggest that Eda-A1 signaling has multiple roles in ectodermal organ development regulating their initiation, morphogenesis, and differentiation. The function of Edar pathway during the first steps of organogenesis will be discussed in detail.

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CYTOKINES AND IMMUNE REGULATION

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The concept of a receptor classically includes ligand recognition (usually with high affinity and specificity) and signaling. The type II IL1 receptor binds IL-1β with high affinity but is not part of signaling receptor complexes. By sequestering the ligand in membrane-bound or soluble form, and by acting as a dominant negative for the accessory protein, it inhibits IL-1. After definition of the IL-1RII as a decoy receptor, decoy receptors have been identified among members of the IL-1, TNF and IL-10 family. Moreover, recent results suggest that functionally uncoupled, decoy receptors can be generated in the chemokine receptor family and that silent, nonsignalling chemokine receptors can act as decoys. Therefore, decoy receptors are a general strategy to regulate inflammatory cytokines and chemokines.

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XSCID AND BEYOND: THE BIOLOGY OF INTERLEUKINS 2, 4, 7, 9, 15, AND 21

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The most common form of severe combined immunodeficiency, XSCID, results from mutations in the common cytokine receptor γ chain, γ_c, which is shared by IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. In XSCID and the related Jak3 deficiency, the defective T-cell and NK-cell development can respectively be attributed to defective IL-7R vs. defective IL-15 signaling. Many γ_c-dependent cytokines, activate Stat5 proteins, which are essential for NK-cell development and contribute to T-cell development and $CD8^+$ T-cell homeostasis. To clarify the basis for XSCID, we compared the gene expression profiles of IL-2, IL-4, IL-7, and IL-15. IL-2, IL-7, and IL-15 each induced a highly similar set of genes in T cells, whereas IL-4 induced distinct genes, correlating with differential STAT protein activation by this cytokine. Both induced genes such as dual specificity phosphatase 5 and repressed genes such as IL-7 receptor were identified and will be discussed. To investigate the B-cell defect in XSCID, we generated IL-21R knockout mice. These mice have diminished IgG1 and elevated IgE, while mice lacking both IL-4 and IL-21R exhibit a dysgammaglobulinemia with a severely impaired IgG response. Thus, IL-4 and IL-21 cooperatively influence B-cell function, suggesting that defects in these cytokines may explain the XSCID B-cell defect. Thus, the combined actions of IL-7, IL-15, IL-4, and IL-21 appear to account for the T-cell, NK-cell, and B-cell defects in XSCID.

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DISCOVERY OF INTERLEUKIN 28 AND 29, INTERFERON-LIKE CYTOKINES THAT SIGNAL THROUGH A NOVEL CLASS II CYTOKINE RECEPTOR IL-28R

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Using a method that combines multiple computational techniques to detect families of uncharacterized proteins from human genomic sequence, we have identified a family of human cytokine genes that are distantly related to type I interferons. These new cytokine genes, IL-28A, IL-28B and IL-29, are distinct from the known type I interferon genes in several respects including their chromosomal localization and gene structure. However, like type I interferons, gene expression analysis indicates that IL-28 and IL-29 are inducible by viral infection and double-stranded RNA. In addition, exposure of a variety of cell types to purified recombinant IL-28 or IL-29 leads to induction of interferon-stimulated genes resulting in an antiviral state in those cells. Unlike type I interferons, IL-28 and IL-29 do not interact with the type I interferon receptor but instead interact with a novel heterodimeric class II cytokine receptor consisting of IL-10 receptor beta and an orphan class II receptor chain, designated IL-28 receptor alpha. Semi-quantitative RT-PCR analysis of IL-28Rα expression indicates a more limited expression pattern than that seen with the type I interferon receptor. Signal transduction studies indicate that the IL-28R signals through the JAK/STAT pathway in a manner similar to type I interferons. The in vitro and in vivo biological activities of IL-28 and IL-29 suggest they may serve as an alternative to type I interferons in providing immunity to viral infection.

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DIFFERENTIAL ROLES OF TLR2 AND TLR4 IN MACROPHAGE ACTIVATION AND IMMUNITY

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We and others have previously reported that Toll-like receptor-2 (TLR2) agonists induce expression of a more limited repertoire of pro-inflammatory genes than TLR4 agonists. Murine macrophages stimulated with the TLR4 agonist, *E. coli* lipopolysaccharide (LPS), induced signal transducer and activator of transcription 1 (STAT1) tyrosine phosphorylation that was secondary to the autocrine/paracrine action of interferon (IFN)-beta, an immediate early gene. In contrast, TLR2 agonists failed to activate IFN-beta gene expression. TLR4-induced IFN-beta mRNA was found to be MyD88- and PKR (double-stranded RNA-dependent protein kinase)-independent, but TIRAP (Toll/interleukin-1 receptor domain-containing adapter protein)/Mal (MyD88-adapter-like)-dependent. Collectively, these findings provide a putative mechanistic basis for differential patterns of gene expression activated by TLR4 and TLR2 agonists. Additional studies have examined the activation of TLR-dependent signalling pathways leading to STAT1 serine phosphorylation, PI-3-kinase and Protein Kinase C-delta (PKC-delta) activation. Inhibitors of p38 MAP kinase and PKC-delta, but not PI-3-kinase, were found to block the activation of STAT1 serine phosphorylation by LPS. Lastly, our data strongly suggest that a common signalling pathway mediates the activation of PI-3K by TLR2 and TLR4, whereas distinct pathways downstream of these TLR proteins activate PKC-delta.

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LYMPHOTOXIN INDUCED LYMPHOID ORGAN NEOGENESIS ELUCIDATES MECHANISMS OF LYMPH NODE DEVELOPMENT

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Lymphotoxin α (LTα) and LTαβ play crucial, non-redundant roles in inflammation and LN development. LN function depends on T and B cell compartmentalization, antigen presenting cells, and high endothelial venules (HEV) expressing mucosal addressin cell adhesion molecule (MAdCAM-1) and peripheral node addressin (PNAd), ligands for naïve cell entrance into LN. Luminal PNAd expression requires a HEV restricted sulfotransferase (HEC-6ST). To investigate LTαβ's mechanisms in lymphoid organogenesis, mice simultaneously expressing LTα and LTβ under rat insulin promoter II (RIP) control were compared with RIPLTα mice in a model of lymphoid organ neogenesis and with $LT\beta^{-/-}$ mice. RIPLTβ mice expressed LTβ in the pancreas with no cell recruitment. In contrast, RIPLTαβ pancreata exhibited massive intra-islet mononuclear infiltrates that differed from the more sparse RIPLTα peri-islet cell accumulations; separation into T and B cell areas was more distinct, expression of lymphoid chemokines (CCL21, CCL19, and CXCL13) was more intense, and L-selectin$^+$ cells made up a higher proportion of the infiltrate. In contrast to the predominant abluminal pattern of $LT\beta^{-/-}$ MLN HEV and RIPLTα pancreatic infiltrates, PNAd was expressed pericellularly at the luminal and abluminal aspects of RIPLTαβ HEV, coincident with HEC-6ST. These data highlight distinct roles of LTα and LTαβ in lymphoid organogenesis supporting the conclusion that LTαβ regulates PNAd through HEC-6ST.
LTα and LTαβ regulate gene expression through activation of transcription factors, including NFκb. Data from several groups suggest that the LTαβ complex employs an alternative NIK-Ikkα NFkB pathway. *In vivo* and *in vitro* models of LT signaling allow us to investigate this point particularly with regard to gene expression in lymph node stromal cells and HEV.

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DEVELOPMENT OF NOVEL ANTI-RESORPTIVE THERAPIES TARGETING THE RANKL-RANK SIGNALING PATHWAY

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The discovery of the TNF/TNFR-related osteoprotegerin (OPG)/RANKL/RANK axis and its central role in osteoclast biology has provided the basis for the generation of therapeutics. Transgenic mice expressing osteoprotegerin hepatically (apoE) had normally-shaped, dense bones histologically consistent with osteopetrosis. Conversely, the OPG k/o exhibited profound high turnover osteopenia with spontaneous fractures. The ligand for OPG was identified in a 32D cell library and was identical to RANKL, a ligand for the TNFR family member RANK. RANKL promotes the differentiation, activation, and survival of osteoclasts through RANK. Knockouts for both RANKL and RANK yielded mice with severe osteopetrosis in addition to uncovering a role for this axis in lymph node and mammary gland development. Overall, RANKL binding proteins have shown beneficial effects in rodent models of postmenopausal osteoporosis, hypercalcemia of malignancy, metastatic bone disease, weightlessness, rheumatoid arthritis, and multiple myeloma. Two forms of OPG have entered early clinical trials and both have shown profound antiresorptive activity following single dose exposures and were well tolerated. While the ultimate success of any therapeutic is difficult to predict in early development, this program is an example of how basic discoveries have provided a strategy to target osteoclasts in diseases characterized by excessive bone resorption.

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TNF REGULATION IN CHRONIC INFLAMMATION: THE INVOLVEMENT OF A NEWLY DEFINED POPULATION OF T CELLS

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Whilst blockade of TNFα activity in rheumatoid arthritis is an effective therapy, concern still exists that in the long-term, abrogation may compromise immunity. We have been interested in identifying the mechanism(s) responsible for inducing cytokine production in inflammatory sites, and to determine if this differs from that required as part of an immune response.

We have found that cytokine production in synovial tissue macrophages from RA but not OA patients was T cell dependent. The RA synovial joint T cells resembled 'bystander-activated T cells generated from normal blood over 8 days using a cocktail of cytokines. These bystander-activated lymphocytes *and* RA synovial T cells both induced TNFα production in resting monocytes in a cell-contact dependent manner, which was abrogated by blockage of the transcription factor NF-κB but augmented if PI3 kinase was inhibited.

The bystander-activated lymphocytes consist of both CD3+ve memory T cells but also a significant number of CD3-ve, CD56+ve NK cells. Interestingly both populations of cells are capable of inducing TNFα production in monocytes in a contact-dependent manner.

These data provide strong evidence for the importance of bystander activated lymphocytes in inducing TNFα in chronic inflammatory rheumatoid tissue, and raises the exciting possibility that selective inhibitors of TNFα for chronic inflammatory diseases may be developed.

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TRIGGERING THE INTERFERON ANTIVIRAL RESPONSE THROUGH AN IKK-RELATED PATHWAY

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Virus infection of susceptible cells activates multiple signaling pathways (IKK-NF-κB/JNK/PI-3K-AKT) that orchestrate the induction of cytokines involved in the antiviral and innate immune response. Virus infection also stimulates a virus-activated kinase (VAK) responsible for the C-terminal phosphorylation and activation of interferon regulatory factor 3 (IRF-3) and the closely related IRF-7, both of which are central to IFN production and activation of the antiviral cascade. Recognition of bacterial infection mediated through lipopolysaccharide (LPS) by Toll-like receptor 4 (TLR-4) and double stranded RNA by TLR-3 also induces IRF-3/IRF-7. The C-terminal end of IRF-3 and IRF-7 contains several potential phosphoacceptor sites that are targeted by the VAK activity. Identification of VAK activity is critical to an understanding of the signaling pathways involved in linking the host response to pathogens with the establishment of the antiviral state. We now demonstrate that the IKK-related kinases – IKKepsilon/TBK1 – are components of VAK and specifically phosphorylate the C-terminal residues of IRF-3 and IRF-7. Expression of IKKepsilon or TBK1 was sufficient to induce cytoplasmic to nuclear translocation of 90-95% of IRF-7 and about 40% of IRF-3. Nuclear extracts of IKKepsilon-transfected cells are specifically recognized in immunoblot analysis using an IRF-3 phosphospecific Ser396 antibody. Co-transfection of IKKepsilon alone stimulates reporter gene activities corresponding to RANTES, IFNb, IFNa1 and IFNa4; the combination of IRF-7 and IKKepsilon resulted in a 2000-fold stimulation of the IRF-7-regulated IFNa4 promoter. IKKepsilon mediated activation of the cytokine reporter genes was blocked by co-expression of dominant negative mutants of IKKepsilon or TBK1 and by siRNA targeting. Furthermore, IKKepsilon stimulates expression of the endogenous IRF-3-dependent gene ISG56 and mounts an antiviral response that inhibits VSV multiplication. These studies constitute the first biochemical evidence linking the NF-κB and IRF pathways in the response to pathogens, specifically through a distinct IKK-related pathway.

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SIGNALING BY THE TNF/NGF RECEPTOR FAMILY: TOWARDS NEW LEVELS OF COMPLEXITY

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There is still vast gap between our knowledge the cellular functions that receptors of the TNF/NGF family regulate and our knowledge of the signaling mechanisms that they activate. A wide range of different cellular activities are told to be accounted for by just a few signaling molecules and pathways, with only limited clues of mechanisms that may account for receptor- and cell-specificity in this regulation. This discrepancy can be traced to limitations of the means by which our knowledge of function of the individual signaling molecules has been gained. We shall present new findings of several of the signaling proteins, showing that (a) Caspase-8, an enzyme identified as the initiator of cell death induction by receptors of the family, also serves in a variety of tissues some non-apoptotic roles. (b) NIK, a protein kinase mediating activation of a distinct NF-κB pathway by the LTβR, CD40 and BLyS, also mediates the function of some other receptors of the TNF/NGF family and, in response to some of them also affects the activation of the 'canonical' NF-κB pathway. (c). CYLD, a deubiquitinating enzyme bound both to NEMO/IKKg and to TRAF2 serves to withhold NF-κB activation by signaling proteins that act upstream of the IKK signalosome, and does so apparently in a highly receptor-specific manner.

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ANTI-CYTOKINE THERAPIES FOR INFLAMMATORY DISEASES: PRESENT AND FUTURE

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Cytokines evolved as part of the host-defense system against infection and to assist in repair from injury. IL-1 and TNFα are examples of cytokines that participate in host-defense functions but are also validated targets of specific blockading strategies, particularly for the treatment of inflammatory and autoimmune diseases. The seemingly paradoxical property of these cytokines is due to a transient and limited expression during a self-limiting disease compared to an uncontrolled and persistent expression during a chronic disease. In fact, chronic and progressive cytokine-mediated disease is characterized by unrelenting production of pro-inflammatory cytokines such as IL-1 and TNFα. The role of these cytokines in the severity of inflammatory diseases has been validated by therapies, which specifically prevent their multiple biological activities. IL-15, IL-17 and IL-18 are also targets in rheumatoid arthritis, psoriasis and similar autoimmune diseases. Acute coronary syndromes is a likely target for IL-1 or IL-18. Although there are no major differences in the efficacy between the anti-cytokine agents, treating a chronic disease with an anti-cytokine can lead to reduced host defense functions. Therefore, safety becomes an important consideration in medical decision-making processes. In the case of monoclonal antibodies to TNFα, increased serious and even fatal infections as well as lymphoma risk are being studies for causality. Not all TNF blockers have the same risk or pharmacokinetics. At present, blocking IL-1 appears safer than blocking TNF. It appears that the future of anti-cytokine therapy will include newer cytokine targets, greater numbers of diseases to treat as well as increased numbers of patients suitable for treatment.

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ROLE OF ADAPTOR MOLECULES IN TLR SIGNALLING

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Innate immune response is a first-line defense system in which individual Toll-like receptors recognize distinct pathogen-assoicated molecular patterns (PAMPs) and exert subsequent immune responses against a variety of pathogens. TLRs are composed of an extracellular leucine-rich repeat (LRR) domain and a cytoplasmic domain that is homologous to that of IL-1R family. Upon stimulation, TLR recruits IRAK via adapter MyD88, and finally induces activation of NF-kappaB and MAP kinases. However, the response to TLR ligands differs each other indicating diversity of TLR signaling pathways. Besides MyD88, several novel adaptor molecules including TIRAP/Mal and TRIF have recently been identified. Knockout mice of these adapters demonstrated that differential utilization of these adapter molecules provide specificity in TLR signaling.

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THE IMPACT OF CCR7 AND CXCR5 ON LYMPHOID ORGAN DEVELOPMENT AND ADAPTIVE IMMUNITY

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The adaptive immune response relies on a precise temporal and spatial positioning of lymphocytes within lymphoid and non-lymphoid tissues. Chemokines, either constitutively expressed or induced during inflammation provide a flexible navigation system directing lymphocytes into specific microcompartments. Precision and specificity in this process are achieved by varying patterns of chemokine receptors expressed on the cell surface of lymphocytes in the course of cell differentiation. The chemokine receptors CXCR5 and CCR7 are principal regulators for targeting T cells, B cells, and dendritic cells into secondary lymphoid organs. The analyses of knockout mice have been instrumental to explore the crucial role of these receptors for the compartmentalization of secondary lymphoid organs into functionally separated T and B cell zones. CXCR5 and CCR7 as well as their homeostatic chemokine ligands such as CXCL13, CCL21 and CCL19, have now been shown to closely cooperate in the development of lymphoid organs. In addition, CCR7 and CXCR5 have been identified as useful markers in the classification of functional distinct subsets of T helper cells, which will lead to a better understanding of T cell memory and T cell effector functions.

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THE IDENTIFICATION OF KEY PROTEINS IN THE INNATE IMMUNE SYSTEM BY MUTAGENESIS AND POSITIONAL CLONING.

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In principle, all genes required for a robust innate immune response can be identified by random germline mutagenesis, phenotypic screening, and positional cloning. Using ENU as a mutagen, we have generated germline mutant mice with specific defects of innate immune sensing and response, and have recently identified one of these mutations, Lps2, as a distal frameshift error in the TIR adapter protein Trif. We have determined that Trif is required for LPS sensing as well as poly I:C sensing. It is essential for a type I interferon response to a viral infection in vivo, and mice with the Lps2 mutation are compromised in their response to mCMV infection. They are also resistant to the lethal effect of LPS. Our data indicate that there are two and only two branches of the LPS response pathway: one served by Trif and the other by Mal/Tirap and MyD88. Ablation of both pathways eliminates the LPS response. However, in the absence of Trif, some LPS responses are eliminated while others remain intact. It appears that in some macrophages, a fourth adapter protein (different than MyD88, Mal/Tirap, or Trif) can permit LPS signalling to occur.

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SIGNALING IN THE INNATE AND ADAPTIVE IMMUNE RESPONSE

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The innate immune response is the first line of defense against pathogens. Upon recognition of specific pathogen components various receptors are triggered leading to activation of a number of signaling pathways. The primary line of defense is mediated by the toll like receptors. In this talk I will first discuss a number of these receptors, the ligands they recognize and the consequences of their absence on resistance to microorganisms. Further, intracellular detection systems have been described, such as the NOD family of proteins. Activation of the innate immune system targets transcriptional pathways such as NF-κB and AP-1 and leads to the upregulation of key target genes. Cell surface receptors signal from the environment the presence of infectious agents, cells carrying peptidic fragments of similar agents, and so on. In this talk we will discuss the signaling mechanisms, which are used by cells of the innate immune system in response to ligation of Toll-like receptors and other related receptor systems. Recent studies show both positive and negative regulatory systems, which gate these signaling pathways. Interestingly, several of these systems are utilized in both cells of the innate and adaptive immune system, showing evolutionary conservation of these signaling mechanisms.

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NEGATIVE REGULATORS OF CYTOKINE SIGNALLING

Ann Cornish, Ben Croker, Sam Wormald, Jian-Guo Zhang, Danielle Krebs, Douglas Hilton, Warren Alexander, Robyn Starr, Andrew Roberts, Lorraine Robb, Manuel Baca, Donald Metcalf and Nicos Nicola, The Walter And Eliza Hall Institute Of Medical Research, Parkville, Australia

Cytokines are an integral component of the adaptive and innate immune responses. The signalling pathways triggered by the engagement of cytokines with their specific cell surface receptors have been extensively studied and have provided a profound understanding of the intracellular machinery that translates exposure of cells to cytokine to a coordinated biological response. It has also become clear that cells have evolved sophisticated mechanisms to prevent excessive responses to cytokines. The suppressors of cytokine signalling (SOCS) are a family of cytoplasmic proteins that complete a negative feedback loop to attenuate signal transduction from the hematopoietin class of cytokine receptors. SOCS proteins inhibit components of the cytokine signalling cascade via direct binding or by preventing access to the signalling complex. The SOCS proteins also appear to target signal transducers for proteasomal destruction. Analysis of genetically modified mice in which SOCS proteins are overexpressed or deleted have established that this family of negative regulators has indispensable roles in regulating cytokine responses in cells of the immune system as well as other tissues. Emerging evidence also suggests that disruption of SOCS expression or activity is associated with several immune and inflammatory diseases, raising the prospect that manipulation of SOCS activity may provide a novel future therapeutic strategy in the management of immunological disorders.

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TRAFFICKING OF CELLULAR AND VIRAL CHEMOKINE RECEPTORS

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Chemokines and chemokine receptors play multiple roles in development and in the functions of the immune system. In addition, they have been implicated in viral and tumor-associated pathogenesis. As with other G protein-coupled receptors, the activity of chemokine receptors is intimately linked to their ability to undergo endocytosis and subsequent sorting for re-sensitization and recycling, or down modulation by degradation in lysosomes. The protective effects of chemokines on HIV infection is mediated through chemokine-induced internalization of cell surface chemokine receptors. Recent studies on CCR5 endocytosis and trafficking through endosomes will be discussed.
Some viruses (in particular Herpesviruses) encode one or more chemokine receptor-like proteins. The HCMV protein US28 binds multiple CC chemokines, as well fractalkine. In contrast to most cellular chemokine receptors, US28 is mostly located in late endosomes with a minor fraction (<20%) expressed on the cell surface. The protein is also constitutively active for signaling and endocytosis. This internalization is independent of β-arrestins, but is dependent on the clathrin-mediated pathway. Constitutive endocytosis of US28 may be important in providing a sink for host chemokines and in targeting US28, and two other HCMV heptahelical proteins, for incorporation into virions that assemble on membranes associated with the endocytic pathway.

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REGULATION OF NF-κB FUNCTIONS

Inder Verma Vinay Tergaonkar Qiutang Li, Ricardo Correa Jeanette Ducut, Virginia Bottero, and Roanna Padre, Laboratory of Genetics, The Salk Institute, La Jolla, CA 92037

NF-κB activity is involved in regulation of many biological and pathological processes. Stimulation and activation of NF-κB depend on a complex regulatory network of associated inhibitors and coactivators under the control of kinases and proteases. IKK1 and IKK2 are key kinases in signal-induced phosphorylation of IκB proteins and subsequent NF-κB activation. We investigated the role of IKK1 and IKK2 in the NF-κB activation pathway by utilizing knockout mice lacking the IKK1 and IKK2. We have identified genes specifically regulated by IKK1 in skin and their functions in keratinocyte differentiation are studied. Chemotherapeutic agents are known to simultaneously induce the transcription factors p53 and NF-κB. To investigate a potential link between these opposing pathways, we are analyzing the p53 response in $IKK1^{-/-}IKK2^{-/-}$ MEFs. Our results uncover distinct functions of the highly homologous kinases IKK1 and IKK2 in regulating p53 stability and suggest a mechanism for the acquisition of resistance to chemotherapeutic agents, which activate both NF-κB and p53 in the absence of mutations in p53.
We have explored NF-κB signaling pathway in other species. Apparently, NF-κB cascade signaling is essential for vertebrate development. Deficiency in the expression of IKK and/or NF-κB proteins is involved with aggressive phenotypes like liver degeneration, skin and skeletal defects and hematopoiesis abnormalities. I will discuss the mechanism by which function of NF-κB protein is regulated in response to external signals.

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THE ROLE OF INNATE CYTOKINES IN INFLAMMATION

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Mediators produced by innate immune response cells such as macrophages can profoundly influence adaptive immunity. Recent studies have shown that IL-15 and IL-18 play an influential role in inflammatory response. Here I present recent data illustrating the importance of IL-15 and IL-18 in the induction and perpetuation of chronic inflammation during experimental and clinical rheumatoid synovitis. These findings suggest that antagonists to these cytokines may have potential therapeutic role against organ-specific autoimmune diseases. In a recently concluded Phase I/II clinical trial, a human anti-IL-15 antibody showed positive results for rheumatoid arthritis. Thirty patients who had previously failed to respond to standard disease modifying arthritis drugs (DMARDS) took part in this randomized, placebo controlled dose-escalating study. 65% of patients achieved an ACR20, with 43% of patients reaching an ACR50 and 26% attaining an ACR70. The ACR scale is comprised of objective criteria defined by the American College of Rheumatology (ACR) with an ACR20 as the benchmark for efficacy. The antibody (HuMax-IL-15) was also safe and well tolerated in this trial. Other innate mediators such as ST2 and Toll-like receptors also play an important role in inflammation. The therapeutic potential of these reagents in the experimental murine models of inflammation will also be discussed.

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SIGNALLING MECHANISMS OF TOLL-LIKE RECEPTORS

Brian Foxwell, Kennedy Institute, Imperial College, London/UK

It is now accepted that toll receptors are an important part of the host defence against microbial attack and may play in autoimmunity. In this presentation aspects of the signalling mechanisms mediated by toll receptors will be examined particularly the role of tyrosine kinases. In addition, the prospect that the nature of toll signalling mechanism may alter between different cell types will be discussed.

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DEVELOPMENT AND FUNCTION OF IL-10-PRODUCING REGULATORY CD4$^+$ T CELLS

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There is increasing evidence to support the existence CD4$^+$ T cells subset with regulatory capacity that can downregulate inflammatory pathologies. However, conditions for generating a homogeneous population in vitro of antigen-specific regulatory CD4$^+$ T cells in large numbers has not been described and still represents a major limiting factor. We show that a combination of the immunosuppressive drugs, Vitamin D3 and Dexamethasone, induce mouse and human naïve CD4$^+$ T cells to differentiate in vitro into regulatory T cells. In contrast to previously described in vitro derived CD4$^+$ T cells, these cells produced only IL-10, but no IL-4, IL-5 and IFN-gamma, and furthermore could be expanded in IL-2. The development of these IL-10-producing cells was enhanced and most reproducible when Th1 and Th2 inducing cytokines IL-4, IL-12 and IFN-gamma were neutralized. This population was only induced when the immunosuppressive drugs Vitamin D3 and Dexamethasone were used in combination, together with stimulation of the cells through their TCR, while using these drugs individually did not lead to the generation of such regulatory cells. The development of such IL-10 producing T cells occurred in the absence of APC, IL-10 acting as a positive autocrine factor for these T cells. Furthermore, NF-κB and AP-1 activities were inhibited in these IL-10 producing cells, as well as key transcription factors involved in Th1 and Th2 subset differentiation. These IL-10 producing T cells showed regulatory function in that when adoptively transferred they prevented inflammation in the central nervous system. We now compare properties of these IL-10-producing regulatory T cells with properties of other regulatory T cells described to date.

AIDS

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HIV-1 NEF MEDIATES LYMPHOCYTE ACTIVATION TRIGGERING THE RELEASE OF CYTOKINE AND CHEMOKINE FROM DENDRITIC CELLS

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Immature dendritic cells (DCs) are targets for productive HIV infection, whereas mature DCs are efficient transmitters of HIV to T cells. DCs by clustering and activating T cells, may both activate antiviral immunity and facilitate virus dissemination. The HIV-1 Nef was shown to mimics many pathogenic effects of HIV infection independently of the presence of infectious virus or even other HIV genes.

Here we demonstrate that exogenous Nef is efficiently taken up by immature DCs and triggers a significant increase in the production of IL-1β, IL-12, IL-15, TNF-α and IL-6 by immature DCs, while Nef does not modulate IL-10 secretion. The induction of cytokines production triggered by Nef correlates with the increased immunostimulatory capacity of immature DCs. We find that Nef-treated immature DCs show a significant increase in MIP-1α, MIP-1β and IL-8 production while RANTES and MDC production are not modulated. This has a direct impact on the increased capacity of DCs to form clustrers with allogeneic $CD4^+$ T cells, improving immunological synapse formation.

Cytokine and chemokine mRNA levels are consistent with protein release. The Nef- induced gene activation correlates with the activation of the NF-kB trancription factor.

Our results highlight how Nef may enhance lymphocyte recruitment and activation, thus fostering virus dissemination.

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MYCOBACTERIUM TUBERCULOSIS ACTIVATED ANERGIC $CD8^+$ T CELLS INHIBIT HIV REPLICATION VIA AN IL-10 DEPENDENT MECHANISM

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Tuberculosis (TB) TB and AIDS are the largest causes of infectious disease death globally and the co-infection with HIV-1 and *Mycobacterium tuberculosis* (MTb) is increasingly common in the developing world. In this study we investigated the impact of recall responses to mycobacterial antigens on HIV-1 replication. Using PBMC from former TB patients who displayed either a persistently positive (responsive) or negative (anergic)- skin reaction to intradermal injection of protein-purified derivative (PPD) and an impairment of T cell activation to PPD, we investigated the impact of response to MTb antigen on HIV-1 replication *ex vivo*. We show that antigen-specific responses to mycobacterial recall antigens significantly inhibit HIV-1 replication in the PPD-anergic PBMC as compared to PPD-responsive PBMC. In response to PPD stimulation, anergic donors PBMC contain a disproportionate expansion of $CD8^+$ T cells and higher levels of interleukin-10 (IL-10) than PBMC from PPD-responsive donors. Depletion of $CD8^+$ cells and blocking of IL-10 significantly increases HIV-1 replication in anergic cultures. Supernatants from anergic cultures also contain lower levels of interleukin-2, interferon-γ, and TNF-α than PPD-responsive cultures. Therefore, an immunosuppressive host specific response to TB recall antigens inhibits HIV-1 replication.

ANTAGONISTS

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DESIGNER PROTEINS TO SPECIFICALLY ACTIVATE OR INHIBIT SPECIFIC CYTOKINE RESPONSES

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Cytokine receptors exist in membrane bound and soluble form. While most soluble receptors are antagonists some soluble receptors are agonists like soluble receptors of the IL-6 cytokine family. *In vivo,* the IL-6/soluble IL-6R complex stimulates several types of target cells not stimulated by IL-6 alone, since they do not express the membrane bound IL-6R. This process has been named transsignaling.

We have constructed fusion proteins consisting of agonistic soluble cytokine receptors and cytokines. The fusion protein of soluble IL-6R and IL-6 named Hyper-IL-6 has been shown to be 100-1000 times more active than the separate natural proteins. Furthermore, we have shown that soluble gp130 is the natural inhitor of IL-6/soluble IL-6R complex responses. Soluble gp130 can be used to discriminate between gp130 responses via membrane bound and soluble IL-6R responses.

Using Hyper-IL-6 and related designer cytokines we have shown that hematopoietic cells and neural cells are regulated by the 6/soluble IL-6R complex. Moreover, using the soluble gp130 protein we demonstrate that in several chronic inflammatory diseases such as chronic inflammatory bowl disease and peritonitis, transsignaling via the soluble IL-6R complexed to IL-6 is a crucial point in the transition from the acute to the chronic state of the disease.

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A THERAPEUTIC HTNFα VACCINE INDUCES ANTI-TNFα ANTIBODIES THAT INHIBIT SIGNALLING THROUGH THE TNFα-TNF RECEPTOR PATHWAY

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Abstract Text: TNFα is a pleuripotent cytokine with a central role in inflammation. Direct anti-TNF targeting therapy, consisting either of monoclonal antibodies or soluble TNF receptors, has proven successful in halting inflammatory diseases e.g. rheumatoid arthritis, Crohn's disease and ankylosing spondylitis. Development of anti-product antibodies to the anti-TNF agents administered is often associated with unresponsiveness to the treatment. We have developed a novel TNFα pharmaccine (therapeutic vaccine) based on the patented AutoVac™ technology to effectively neutralize endogenous TNFα. The immune response is driven by the TNFα AutoVac™ protein and not by endogenous TNFα, and needs re-immunisations to sustain a high antibody response. TNFα AutoVac™ proteins were designed to preserve the TNFα structure and were expressed in *E. coli* as soluble proteins. TNFα AutoVac™ is active (toxic) against TNFRI expressing cell lines and has matching biophysical characteristics, as investigated by heat denaturation, CD and light scattering, with wtTNFα. In order to remove the cytotoxicity from our vaccine, a single-point mutation was introduced in TNFα AutoVac™. Antisera produced by vaccination with TNFα AutoVac™ inhibits the *in vitro* binding of TNFα to TNF receptors, promising a potential role as a novel kind of anti-TNFα therapy in inflammatory diseases.

APOPTOSIS

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INTERLEUKIN-6 AND ONCOSTATIN M SENSITIZE FOR TNF-MEDIATED CELL DEATH IN EPITHELIAL CELLS

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Interleukin-6 was described as an autocrine growth factor for various cell types. STAT3, a major signal transducer of IL-6, has previously been implicated in the prevention of apoptosis. We show that IL-6 enhances susceptibility to TNF-mediated cell death in epithelial cell lines. Sensitization to TNF apoptosis was not only induced after IL-6 stimulation but also after application of the gp130-activating cytokine oncostatin M. Both cytokines strongly activated STAT3 in these cells. As judged by RNase protection analysis, enhanced TNF-mediated apoptosis was not due to the up-regulation of the TNF receptor or associated factors. IL-6 and OSM did not lead to a general enhancement of TNF functions. Thus, neither NF-κB activation, nor TNF-mediated induction of the chemokine IL-8 involving a variety of transcription factors were affected. Also, IL-6 and OSM did not change the expression of caspases involved in TNF-mediated cell death. This was substantiated by the fact that the cytokines did not sensitize for Fas-mediated cell death demonstrating that they had no effect on the apoptosis effector pathway common for Fas and the TNF receptor. In conclusion, IL-6 and OSM specifically sensitize epithelial cells for TNF-mediated apoptosis. The molecular mechanism of sensitization is currently investigated.

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INVOLVEMENT OF IL-1 SYSTEM IN APOPTOSIS OF MURINE HIPPOCAMPAL GRANULE NEURONS INDUCED BY TRIMETHYLTIN INJECTION

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The dentate gyrus granule neurons of the hippocampus are well recognized as relatively resistant to injury induced by pathogenic stimuli. In our studies we applied the rare model of selective degeneration of murine hippocampal granule cells, caused by trimethyltin (TMT) administration in dose 2.5 mg/kg b.w.. Granule cell death bore the apoptotic features: chromatin condensation, oligonucleosomal DNA fragmentation and caspase-3 activation, evident as early as 1 day after intoxication. Using RPA method we found the increase of interleukin-1beta (IL-1beta) and IL-1 receptor antagonist (IL-1ra) mRNA in the hippocampus 3 days after treatment, confirmed on the protein level using Western blot method. Immunocytochemical studies showed that the cellular source of both cytokines was ameboid microglia, activated in the region of degeneration. However, the distribution of IL-1beta and IL-1ra was distinct within the granule layer. Whereas IL-1beta-positive cells were mainly localized in the crest of dentate gyrus, where the highest number of apoptotic neurons appeared, IL-1ra-positive microglia was practically restricted to the remaining dentate regions. Temporal and spatial relation between apoptosis and expression of IL-1 family proteins suggests the active involvement of IL-1beta in neuronal apoptosis, whereas IL-1ra could be engaged in protection of spared granule cells.

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TGF-β1-INDUCED APOPTOSIS IS MEDIATED BY CASEIN KINASE 2 AND P53 AND IS INDEPENDENT OF MAP KINASES

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Various cytokines regulate apoptosis through distinct signaling cascades that involve different cytosolic kinases and target different nuclear transcription factors. Studies have shown that TNF-α and IL-6 protect against apoptosis and that these cytokines act via MAPKs and the transcription factor, NF-κB. In contrast, TGF-β1 induces apoptosis. Using the mouse epithelial cell line, SVEC4-10, and normal fibroblast cells, L929, we studied the TGF-β1-induced cell death and explored the role of p53 in this pathway. Treatment with TGF-β1 resulted in phosphorylation and increased DNA binding activity of p53. In these studies, activation and phosphorylation of p53 were monitored by western blot using pS392 and pS15-specific antibodies and by phospho ELISAs. Treatment with 5,6-dichloro-1-beta-D-ribofuranosyl-benzimidazole, a specific inhibitor of CKII, reversed the transcriptional activity of p53, suggesting that CKII mediates TGF-β1-induced apoptosis by regulating the phosphorylation state and the DNA binding affinity of p53. Inhibitors of MAPKs, SB203580 and PD98059, failed to reverse the TGF-β1-associated p53 activation and subsequent apoptosis, suggesting that the TGF-β1 mechanism is independent of the MAPKs. Bcl-2 ELISA and Caspase-9 assay were used to correlate TGF-β1-mediated apoptosis and increased levels of Bcl-2 and caspase-9 activity. We conclude that TGF-β1 induces apoptosis by activating CKII, which leads to site-specific phosphorylation of p53 and its binding to DNA.

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TUMOR NECROSIS FACTOR RECEPTOR-ASSOCIATED FACTOR (TRAF) 2 AND TRAF5 ARE ESSENTIAL FOR IL-15 RECEPTOR ALPHA-MEDIATED SIGNALING

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The α chain of the IL-15R (IL-15Rα) serves as the specific, high affinity receptor for IL-15, a pleiotropic cytokine with many IL-2-overlapping activities and a potent anti-apoptotic function. We analyzed the involvement of TRAF adapter proteins in the signaling pathways downstream of murine IL-15Rα using co-transfection and immunoprecipitation experiments. Of the six known TRAF family members, TRAF2 and TRAF5 associate and efficiently compete for binding to the IL-15Rα chain. Like most other TRAF-interacting receptors, overexpression of IL-15Rα activates the transcription factor NF-κB. Co-overexpression of IL-15Rα with TRAF2 and, to a lesser extent, with TRAF5 results in synergistic activation of NF-κB. The introduction of a dominant negative mutant of TRAF2 suppresses NF-κB activity in a dose-dependent manner, suggesting a potentially more important role for TRAF2 in the post-receptor signaling. Mutational analysis reveals that substitution of first (E240), second (E242) or both glutamic residues to threonine in the putative TRAF-binding ^{239}VEVET^{243} motif of the IL-15 receptor alpha intracellular tail fully abrogates binding of TRAFs as well as NF-κB activation. Furthermore, such mutations inhibit the IL-15 ability to support the survival of IL-15Rα-transfected BA/F3 cells clones. These results indicate that TRAF2 and TRAF5 bind to the IL-15Rα via a highly conserved consensus motif and may mediate the diverse biological effects of IL-15, including its anti-apoptotic properties.

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TAURINE ATTENUATES CD3/IL-2-INDUCED APOPTOSIS VIA MODULATION OF FASL EXPRESSION IN A MODEL OF T-CELL AICD

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The use of IL-2 in immunotherapy of cancer is impeded by the induction of lymphopenia. Physiologically, IL-2 plays a pivotal role in the maintenance of immune homeostasis through induction of T-cell Activation-Induced Cell Death (AICD). In light of its role in AICD we suggest that AICD is the mechanism underlying the loss of T-cells during IL-2 therapy. We have previously increased the therapeutic index of IL-2 through combination with the amino acid taurine, and report here that taurine attenuates AICD in both Jurkat cells and peripheral blood $CD4^+$ T-cells (PBLs).

T-cells (Jurkat & PBLs) were stimulated with anti-CD3 mAb and IL-2. Some groups were preloaded with taurine. FasL, IL-2R and apoptosis were measured by flow cytometry. FasL mRNA was assessed via mRNA ELISA.

Stimulation of Jurkat and PBLs with CD3/IL-2 induced apoptosis, mediated in part by FasL. Taurine significantly reduced apoptosis through modulation of FasL protein and mRNA expression.

Taurine attenuates CD3/IL-2-induced T-cell AICD through a FasL-dependent mechanism. Induction of AICD may underlie the lymphopenia induced during IL-2 therapy, and our results indicate that taurine may be of benefit in this context.

AUTOIMMUNITY

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ELEVATED MATRIX METALLOPROTEINASE-9 IN PATIENTS WITH SYSTEMIC SCLEROSIS

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Objective: To determine the expression of matrix metalloproteinase 9 (MMP-9) and its clinical significance in systemic sclerosis (SSc).
Methods: The levels of MMP-9 and tissue inhibitor of metalloproteinase (TIMP)-1 were measured in sera and culture supernatants of dermal fibroblasts from SSc patients by an enzyme linked immunosorbent assay (ELISA) and/or gel zymography. Serum TGF-β levels as well as clinical and laboratory findings of SSc patients were investigated at the time of sampling.
Results: The patients (n = 42) with SSc had higher levels of MMP-9, TIMP-1, and the ratio of MMP-9 over TIMP-1 in sera than healthy controls (n = 32). The MMP-9 levels were significantly higher in the diffuse type (n = 23) than the limited type of SSc (n = 19). The serum concentrations of MMP-9 correlated well with the degree of skin involvement, as determined by Rodnan score, and serum TGF-β levels. In particular, dermal fibroblasts from SSc patients produced a higher quantity of MMP-9 compared to those from healthy controls when they were stimulated with IL-1β, TNF-α, and TGF-β. Such an increase in MMP-9 production was partially blocked by the treatment with cyclosporin A.
Conclusion: The serum MMP-9 levels were elevated in SSc patients and correlated well with skin scores. The increased MMP-9 levels may be explained by the overproduction from dermal fibroblasts of SSc. These data suggest that the enhanced production of MMP-9 may contribute to fibrogenic remodeling during the progression of skin sclerosis in SSc.

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TGFβ1 PREVENTS AUTOIMMUNE RESPONSE BY MAINTAINING $[Ca^{2+}]i$ HOMEOSTASIS IN T CELLS

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Transforming growth factor *beta*1 (TGFβ1) is a polypeptide growth modulatory and differentiation factor involved in many biological processes including immune homeostasis and self-tolerance. *Tgfb1* knockout mice die at weaning age from a multifocal autoimmune disorder. Elimination of T cells but not B cells eliminates the autoimmunity. Immature *Tgfb1*$^{-/-}$ thymic T cells are hyper-responsive to mitogenic stimulation, whereas mature splenic T cells are hypo-responsive. Immature DP and SP thymocytes exhibit elevated $[Ca^{2+}]i$ levels. In response to anti-CD3, Ca^{2+} flux is increased in *Tgfb1*$^{-/-}$ $CD4^+$ SP thymocytes, but not affected in *Tgfb1*$^{-/-}$ DP cells. Mature *Tgfb1*$^{-/-}$ splenic T cells are already activated *in vivo* as evidenced by elevated basal $[Ca^{2+}]i$ levels, reduced Ca^{2+} flux upon stimulation, decreased CD3 expression, increased LFA-1 expression, and increased cell size. Our data demonstrate that the hypo-responsiveness of *Tgfb1*$^{-/-}$ splenocytes is due to prior activation of T cells resulting from deregulated $[Ca^{2+}]i$ levels. These findings suggest that TGFβ1 functions to inhibit aberrant T cell expansion and autoimmunity by maintaining $[Ca^{2+}]i$ levels low enough to prevent a mitogenic response in the absence of optimal stimulation.

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ANTI-OXIDANTS DOWN-REGULATE THE EXPRESSION OF PROINFLAMMATORY CYTOKINES IN RAT AUTOIMMUNE DIABETES

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Type 1 diabetes (T1D) is a disease that results from autoimmune destruction of the insulin-producing pancreatic β-cells. The production of proinflammatory cytokines, followed by release of reactive oxygen and nitrogen intermediates, represents a key event in the autoimmune islet damage. Anti-oxidants are powerful inhibitors of NF-κB, which has an important role in T cell activation and production of various proinflammatory mediators. We therefore tested the possible therapeutic value of two anti-oxidants, butylated hydroxyanisole (BHA) and pyrrolidine dithiocarbamate (PDTC), in the animal model of diabetes induced in susceptible DA rats by multiple low doses of streptozotocin (MLD-SZ, 20 mg/kg/day for 5 days). Administration of either BHA, or PDTC (50 mg/kg/day for 7 days), after finishing MLD-SZ injections, attenuated both the development of hyperglycemia and insulitis. *Ex vivo* analysis revealed that BHA treatment reduced the proliferation of autoreactive lymphocytes and down-regulated their adhesion to endothelium. In addition, BHA markedly attenuated the production of proinflammatory cytokines IL-1β and TNF-α by both islets of pancreas and peritoneal macrophages. In parallel, macrophage release of cytotoxic oxygen and nitrogen intermediates $O_2^{\cdot-}$ and $NO^{\cdot}$, respectively, was significantly inhibited. Finally, BHA treatment reduced intrapancreatic expression of inducible NO synthase and consequent production of $NO^{\cdot}$ by pancreatic islets. Together, these data indicate that antioxidants might be a feasible therapeutic tools to interfere with development of autoimmune diabetes at multiple levels, including lymphocyte proliferation and adhesion, as well as the production of proinflammatory and cytotoxic mediators. Thus, antioxidant treatment may not only impede the effectory mechanisms that cause β-cell death, but could also interfere with the development of autoreactive T cells and macrophage proinflammatory activity in diabetes.
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TREATING RHEUMATOID ARTHRITIS WITH ANTI-INTERFERON-γ AND ANTI-TUMOR NECROSIS FACTOR -α IN A RANDOMIZED, CONTROLLED TRIAL

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Objective: To assess the potential of anti-IFN-γ and anti-TNF-α in treating RA in a randomized, double-blind, placebo-controlled trial.
Methods: 55 RA patients (pts) who failed previous treatment with at least one DMARD were randomly assigned to receive IM injections of anti-IFN-γ (20 pts), anti-TNF-α (20 pts) or placebo (15 pts). Primary outcome measures were ACR 20%, 50%, and 70% response criteria.
Results: By day 28 both anti-IFN-γ and anti-TNF-α groups showed sustained significant improvement in all clinical measures. Ultrasound analysis showed that only anti-IFN-γ exerted significant reduction of the thickness of the inflamed synovial membrane. ACR maximal response rate was significantly greater in pts treated with anti-IFN-γ. By day 28 no pt in the anti-TNF-α group maintained the 70% improvement achieved previously, whereas the number of ACR 70% responders in the anti-IFN-γ group doubled by this time in comparison with results on day 7 and amounted to 6 of the 17 pts completing the trial. Lupus-like syndrome, Quincke's edema, laryngitis and stomatitis occurred only in the anti-TNF-α recipients (1 pt each).
Conclusion: This double-blind study confirms the beneficial effects of anti-IFN-γ in treating acute RA. The degree of improvement in pts treated with anti-IFN-γ was comparable to that in pts receiving anti-TNF-α and in some aspects was superior to it.

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BLOCKADE OF THE INFLAMMATORY CYTOKINE, INTERLEUKIN 21, REDUCES CLINICAL DISEASE IN ANIMAL MODELS OF ARTHRITIS

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Interleukin 21 (IL-21), a cytokine related to IL2 and IL-15, is secreted by activated T cells and is a powerful immune mediator with inflammatory effects. The receptor for IL21 (IL21R) is most homologous to the IL2Rβ and IL-4Rα chains, and associates with γc upon ligand binding. IL21R is constitutively expressed in B and T lymphocytes, NK cells, macrophages, and dendritic cells and its expression is upregulated by IL21. We investigated the role of the IL21 pathway in animal models of Rheumatoid Arthritis (RA). *In situ* hybridization to paws of DBA/2 mice with Collagen Induced Arthritis revealed elevated expression of IL21 mRNA in lymphocytes, and elevated expression of IL21R in macrophages and synoviocytes in the inflammatory infiltrates. Administration of murine IL-21 at the time of disease onset resulted in exacerbation of clinical signs of CIA. IL21RFc was used to neutralize endogenous IL21 in the Adjuvant Induced Arthritis model in Lewis rats. Therapeutic treatment with murine IL-21RFc ameliorated disease in this model. In human cells, IL21 induced increased expression of inflammatory cytokines and chemokines in primary synoviocytes from RA patients with active disease. These data suggest that blockade of the IL21 pathway might be an effective treatment for Rheumatoid Arthritis.

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EVIDENCE FOR REGULATION OF IFNγ EXPRESSION BY TNFR1 SIGNALLING IN EAE

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TNF plays a complex role in multiple sclerosis and its animal model, experimental autoimmune encephalomyelitis (EAE). We examined cytokine expression levels in mice deficient in the type 1 TNF receptor (TNFR1KO). TNFR1KO mice are resistant to MOG_{35-55}-induced EAE, developing less severe clinical disease with delayed onset compared to wild-type mice. Despite this milder disease phenotype, higher levels of IFNγ and IL-12p40 mRNA were observed in the spinal cord of TNFR1KO mice. We hypothesized that the elevated levels of these pro-inflammatory cytokines was due to TNF-regulated interaction between encephalitogenic T cells and antigen-presenting cells. In a co-culture paradigm, stimulation of MOG-reactive T cells by TNFR1KO macrophages induced higher levels of IFNγ mRNA expression than wild-type macrophages, suggesting a role for TNFR1 signalling in regulation of IFNγ expression. However, there were no intrinsic differences between wild-type and TNFR1-deficient T cells or macrophages, as shown by equivalent responses to anti-CD3 or anti-CD40 stimulation, respectively. These data suggest that the interaction of TNFR1KO macrophages with T cells results in higher IFNγ expression, perhaps through greater release of IFNγ-inducing cytokines, such as IL-12, IL-18 and IL-23.

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COMBINED PROTEOME- AND GENOME ANALYSIS REVEAL GALECTIN-3 AS A CANDIDATE PROTEIN IN TYPE 1 DIABETES PROTECTING AGAINST THE TOXIC EFFECT OF CYTOKINES

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Identifying components of the molecular processes involved in complex traits has proven difficult. Here, we propose a multifaceted approach to extend our understanding of the pathogenesis of type 1 diabetes mellitus (T1D) at the molecular level. We combine proteome, transcriptome and genome analyses to obtain a global analysis of data from cytokine-exposed pancreatic islets, transplantation studies and genetic analyses. The potential of this combined approach is illustrated by investigations of galectin-3. Analyses of human islets and the rat insulinoma (RIN) β-cell line demonstrated increased expression of galectin-3 following cytokine exposure. Functional analyses demonstrated that over expression of galectin-3 protected β-cells against cytokine induced apoptosis, in part through inhibition of JNK signaling. Islet-cell galectin-3 expression *in vivo*, in a transplantation model of T1D, displayed significantly changed expression patterns over time. Genetic analysis demonstrated linkage between T1D and the galectin-3 gene, and several polymorphisms were identified with evidence of association to T1D.
In conclusion, the data support the view that the combined proteome-transcriptome-genome approach represents a valuable tool in studies attempting to identify genes encoding proteins relevant to the pathogenesis of complex diseases as T1D. One such protein. galectine-3 represents a natural, although insufficient protective mechanism in cytokine exposed β-cells.

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TRANSCRIPTIONAL PROFILING OF MURINE COLLAGEN INDUCED ARTHRITIS

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Rheumatoid arthritis (RA) is a complex autoimmune disorder, manifested by inflammation, angiogenesis, hyperplasia, and tissue matrix erosion. Murine collagen induced arthritis (CIA) is a commonly accepted model for the study of RA. Affymetrix Gene Chip™ technology was used to profile changes in gene expression that occur in the paws of mice with CIA. Individual paws were scored for disease on a scale of 0-4, where 0 is no disease, and 4 is maximal edema and inflammation. The number of increasing genes as well as relative levels of expression correlated with paw score, from a low of 23 genes at score 1 to 461 genes at score 4. Most genes, such as CD14, only show up in scores 3 and 4. Genes associated with inflammation such as: Il-1β, Csf1r (colony stimlulating factor 1 receptor), Csf3r (colony stimlulating factor 3 receptor), CD14, S100A9 were observed. Angiogenic genes: Vcam1 (vascular cell adhesion molecule 1), angiopoietin-related, and thymosin beta 10 were increased. Genes associated with mitogenesis, Hck (hemopoietic cell kinase). Genes associated with matrix degradation: MMP3 (matrix metalloproteinase 3), MMP9, MMP13, and Cathepsin K. Data from these experiments may be used to identify genes that are associated with the pathogenesis of rheumatoid arthritis.

CANCER

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INFLUENCE OF PRO-INFLAMMATORY CYTOKINES ON THE ADHESION OF HUMAN PANCREAS CARCINOMA CELLS TO MICROVASCULAR ENDOTHELIUM

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Introduction: Surgical trauma provokes an inflammatory response with secretion of cytokines. We hypothesise that pro-inflammatory cytokines play a crucial role in enhanced tumour cell adhesion in the lung after surgical trauma by upregulating adhesion molecules on microvascular endothelium.
Methods: In a reproducible *in vitro* human tumour cell adhesion model the adhesion of 3 pancreas carcinoma cell lines (Panc-1, Miapaca and Bxpc-3) to monolayers of human microvascular vein endothelial cells of the lung (HMVEC-L) was assessed. To study the influence of cytokines, monolayers of HMVEC-L were pre-incubated with IL-1β, TNF-α and IL-6 for varying times and with varying concentrations. Enzyme immune assay (EIA) was performed to assess adhesion molecule upregulation on HMVEC-L.
Results: Basal adhesion is 17% for Panc-1, 6% for Miapaca and 33% for Bxpc-3. Stimulation with IL-1β and TNF-α, but not with IL-6 significantly enhances the adhesion of the 3 tumour cell lines ($p < 0,01$), which is time- and concentration dependent. Maximal adhesion occurs after 12 hours preincubation. EIA shows increased expression of E-Selectin, ICAM-1 and VCAM-1 on HMVEC-L after stimulation with IL-1β and TNF-α, but not with IL-6. This enhancement is time- and concentration dependent and correlates with the enhancement in tumour cell adhesion.
Conclusion: IL-1β and TNF-α, but not IL-6 are able to increase tumour cell adhesion to HMVEC-L and therefore it is possible that these cytokines are responsible for enhanced tumour take after surgical trauma. Because the time dependency, the increased adhesion is likely to be due to upregulation of adhesion molecules. Insight in the mechanisms of enhanced tumour take after surgical trauma may lead to the development of strategies to prevent post-surgical metastases.

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TNF INDUCTION AND NF-κB ACTIVATION BY THE ANTICANCER AGENT DMXAA

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Our investigations into TNF-inducers for cancer therapy has led to the clinical evaluation of DMXAA, a low molecular weight synthetic agent capable of eliciting sustained TNF synthesis within the tumor mass. Since the in vivo action of DMXAA could be clarified by the development of an in vitro model, we have investigated TNF induction in cultured murine splenocytes. DMXAA induced only low amounts of TNF, but the addition of a low concentration of lipopolysaccharide (LPS) that alone did not induce TNF, led to a synergistic 10 fold increase in TNF production above that obtained with DMXAA alone. Deacylated-LPS, phorbol ester or okadaic acid, and lowering the pH of the culture medium could also provide the 'second signal'. NF-κB was activated by DMXAA or LPS alone, and cultures treated with the combination showed increased NF-κB activation over that obtained with either LPS or DMXAA alone. Parthenolide and salicylate dose-dependently inhibited NF-κB activation and TNF production. These studies indicate that optimal TNF production in response to DMXAA involves a complex interaction between perhaps different cell types and signalling pathways that converge on NF-κB activation. In vivo, this may be provided by the tumour microenvironment leading to selective induction of TNF in the tumor tissue.

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PLASMACYTOID DENDRITIC CELLS FROM HUMAN LYMPH NODES INNATELY PRODUCE IL-12 AND IL-10, BUT IN ADVANCED BREAST CANCER SHOW AN INCREASED IL-10:IL-12 PRODUCTION RATIO

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Plasmacytoid dendritic cells constitute a distinct subset of dendritic cells (DC) found in peripheral lymph nodes, but little is known about their function in tumour immunity. Cell suspensions were prepared from tumour draining lymph nodes ($n = 7$) and control lymph nodes ($n = 3$) of women undergoing surgical resection for primary breast cancer and elective surgery for benign conditions respectively. Using four-colour flow cytometry, DC subsets were identified from their phenotypes. The proportions and numbers of cells innately producing IL-10 and IL-12 were also measured from intracellular accumulation of cytokine after blocking with monensin. All flow cytometry data was collected without compensation and compensated off-line using the Winlist algorithm (Verity software). This package also provided the subtraction programme to calculate percentage positive cells and intensity of staining. Plasmacytoid DC from control lymph nodes and those from patients with a good prognosis ($n = 4$) produced relatively more IL-12 than IL-10. Conversely, patients with a poor prognosis ($n = 3$) preferentially produced IL-10. The significance of IL-10: IL-12 ratios between patient prognostic groups in this preliminary study was $p = 0.03$, Mann-Whitney U. Thus, plasmacytoid DC can produce IL-10 and IL-12 and we speculate that a shift towards IL-10 production may promote tumour tolerance in draining lymph nodes.

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INNATE IMMUNE RESPONSE IN IBD-ASSOCIATED MOUSE COLON CANCER MEDIATED BY TGFβ1

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Tgfb1$^{+/+}$, *Tgfb1*$^{+/-}$ and *Tgfb1*$^{-/-}$ mice on a *Rag2*$^{-/-}$ background develop cecum and colon hyperplasia that is associated with a submucosal granulocytic inflammation. By 3 months of age mucinous carcinoma develops in all *Tgfb1*$^{-/-}$ *Rag2*$^{-/-}$ mice, but rarely do the hyperplastic lesions transition to adenoma or carcinoma in *Tgfb1*$^{+/+}$ *Rag2*$^{-/-}$ or *Tgfb1*$^{+/-}$ *Rag2*$^{-/-}$ mice. Consequently, the tumor suppressor role of TGFβ1 occurs early in the development of mucinous colon cancer. Hence, this model provides us the opportunity to determine "susceptibility" factors for early stages of progression in colon cancer. Previous studies demonstrated that TGFβ1 suppresses susceptibility through maintenance of epithelial tissue organization during inflammatory stress, and that the inflammatory stress may be caused, in part, by *Helicobacter hepaticus*. We hypothesized that the innate immune response to enteric pathogens and the subsequent response of the intestinal mucosa to protect against the potential pathogens are different between lesions from animals that are "susceptible" to progression to colon tumors (*Tgfb1*$^{-/-}$ *Rag2*$^{-/-}$), and "nonsusceptible" lesions (*Tgfb1*$^{+/+}$ *Rag2*$^{-/-}$). To test this hypothesis we have used expression profiling to cluster genes involved in innate immune response and mucosal protection that are differentially expressed in the susceptible vs. nonsusceptible hyperplastic lesions. As expected, we have found that in the susceptible lesions there is an enhanced innate immune response. However, even though the normal response to enhanced innate immune activity is to elevate mucosal protection through mucin production, mucin production was actually depressed in the susceptible lesions. Consequently, in the susceptible lesions the response to enteric pathogens leaves the intestinal mucosa less protected against potential pathogens. Further expression profiling suggests that this in turn results in a dysregulation of mucosal tissue integrity similar to what is found in human colon cancer. This work was supported in part by the Mouse Models for Human Cancer Consortium, NIH.

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THE REGULATION OF A CXCL10-CXCR3 AXIS IN NEUROBLASTOMA CELLS

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It was suggested in our previous study on neuroblastoma cells that CXCL12 (SDF-1) potentiates the metastatic process of neuroblastoma cells. The present study identifies CXCL10 (IP-10) as a chemokine that may limit the growth of neuroblastoma cells. The expression of the CXCL10 receptor, CXCR3, was shown to be a general characteristic of neuroblastoma cells. In CXCR3-expressing neuroblastoma cell lines, the exposure to CXCL10 and CXCL11 (I-TAC) resulted in Erk phosphorylation in a $G_{\alpha i}$-dependent manner. CXCL10 inhibited the growth of the NUB6 and SK-NMC neuroblastoma cells. Both NUB6 and SK-NMC neuroblastoma cells migrated in response to bone-marrow derived conditioned medium, but not to CXCL10. Under stress conditions implemented by serum starvation, the membranous expression of CXCR3 was up-regulated on both the NUB6 and SK-NMC cells. Although serum starvation was accompanied by elevated CXCR3 expression, NUB6 and SK-NMC$^{\gamma}$cells did not migrate to CXCL10. Similarly, serum starvations resulted in impairment of migratory responses to bone marrow-derived conditioned medium. Altogether, these results indicate that an IP-10-CXCR3 axis exists in neuroblastoma cells, and suggest that CXCL10 may counteract the activity of tumor-promoting factors, and limit tumor cell growth.

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NOTCH2 INHIBITS ACTIVATION INDUCED CELL DEATH IN B-CELLS

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We have recently shown that deregulation of Notch2 signaling is involved in the overexpression of *CD23a* in B-CLL cells and might be also implicated in their malfunction of apoptosis. Since it has been demonstrated that Notch1 inhibits activation induced cell death (AICD) in T-cells, we investigated the possible effect of Notch2 on this apoptotic mechanism in B-cell lines.
A retroviral vector coding for Notch2IC was constructed in order to transfect the CD23 negative B-cell line BL41. The BL41/N2IC cells expressed *CD23a*, thereby confirming the functionallity of our Notch2IC construct. To test, whether Notch2 has an impact on AICD, the BL41/N2IC cells and the parental cell line BL41 were then treated with B-cell receptor (BCR) stimulating antibodies. While BL41 cells rapidly underwent apoptosis, the Notch2 transduced cells were significant less sensitive to AICD. Since AICD depends on the *de novo* synthesis of NAK1, the BL41 and BL41/N2IC cells were analyzed for the expression of this gene. By RT-PCR analysis, *NAK1* was found to be induced upon BCR stimulation.
In conclusion, our data indicate, that Notch2 is involved in the modulation of AICD in B-cells. This finding might also be relevant to the pathophysiology of B-CLL lymphocytes and may partly explain the mechanisms responsible for their resistance to apoptosis.

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TNF-α ENHANCES THE TUMOUR CELL SENSITIVITY TO THE CYTOTOXIC AND APOPTOSIS-INDUCING ACTION OF ANTITUMOUR DRUGS

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Recently we have demonstrated that in patients with renal carcinoma, therapeutic activity of rHuIFNa2 is independent from its immunomodulating activity. In vitro, IFNα significantly increases cytotoxic and apoptosis-inducing effect of Doxorubicin and 5-FU in tumor cells overexpressing exogenous Bcl2, and this increasing was much moore strong than in control cells. Our new data on T-cell immunity, NK cells activity and concentration of IgG, A and M in the serum of cancer patients treated with TNF-α suggest no significant immunomodulating activity of TNFα, although several patients treated with TNFα did demonstrate some clinical effect. In vitro studies revealed that rTNFα (alnorin) used in 1-100 IU/ml concentration range increases suscepti-bility of L929 and HeLa cells to cytotoxic and apoptosis-inducing activity of Doxorubicin (0.1-1.0 mcg/ml). In L929 cells, ID50 dose of Doxorubicin administrated in combination with 100, 10 or 1 IU/ml of TNFα decreased 10.4, 6.8 and 8.5 fold, respectively. L929 cells treated with the same concentrations of TNFα alone did not undergo necrosis or apoptosis. On the other hand, TNFα dramatically increased cytotoxicity and apoptosis caused by Doxorubicin both in control and Bcl2-overexpressing HeLa cells. Our data suggests that unlike INFα, TNFα increases susceptibility of tumor cells to Doxorubicin via Bcl2–independent mechanism.

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IDENTIFICATION OF NOVEL HUMAN MEMBERS OF THE DUB DEUBIQUITINATING ENZYMES

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The DUB family of deubiquitinating enzymes are haematopœitic specific cytokine inducible immediate early genes. Three members of this family have been identified; DUB-1, DUB-2 and DUB-2A. These genes have been mapped to mouse chromosome 7 and represent members of a deubiquitinating enzymes family that have resulted from a series of tandem duplication events.
We have previously shown DUB-2 is expressed in HTLV-1 transformed T-cells that show constitutive activation of the IL-2 JAK/STAT pathway. DUB-2 expression in Ba/F3 cells prolongs IL-2 induced STAT5 phosphorylation and inhibits apoptosis induced by cytokine withdrawal.
In this study we set out to identify human members of this family by interrogating human sequence databases using the murine DUB-2 cDNA sequence. A number of open reading frames (ORFs) were identified which showed significant homology to DUB-2. It appears that these ORFs have also resulted from a series of tandem duplication events
Expression of these ORFs was examined by multiple tissue Northern blot. Two transcripts of approximately 1.6 and 1.7 Kb were expressed in a tissue specific manner. Expression of the 1.6 Kb transcript was observed in a panel of tumour cell lines.
This data suggests that expression of these human ORFs is contributing to the development of a number of human tumour types.

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THE N-TERMINAL PRO-DOMAIN OF IL-1α COOPERATES WITH H-RAS TO ENHANCE TUMOR GROWTH IN THE TWO-STAGE SKIN CARCINOGENESIS MODEL

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IL-1α is stored in abundance in skin keratinocytes as an active 31 kDa precursor. Cleavage produces the pro-inflammatory 17 kDa C-terminal domain and a 16 kDa N-terminal pro-domain of unknown physiological function. The pro-domain is known to contain a nuclear localization signal and has been reported to transform fibroblastic cells *in vitro*. To examine its *in vivo* function in skin, a stop codon was introduced into human IL-1a to generate a truncated N-terminal pro-domain polypeptide. Expression of the human pro-domain fused to gfp appropriately localized to the nucleus in human as well as mouse keratinocytes in culture. The truncated human protein was linked to the keratin 14 promoter to generate transgenic mice expressing the pro-domain in the basal layer of the epidermis. In two K14/pro transgenic lines, ProA and ProB, the skin was histologically similar to control littermates suggesting that transgene expression did not disrupt normal skin development or induce inflammation. To test the oncogenic potential of the pro-domain in cutaneous epithelial cells, the classical two-stage skin carcinogenesis protocol was applied using the mutagen DMBA for initiation and the phorbol ester TPA for promotion. In the ProB line, which expresses the transgene at a lower level, a significantly higher average papilloma burden was observed early in promotion (week 16; $p < 0.05$) suggesting that transgene expression enhanced the outgrowth of initiated cells. For the higher expressing ProA line, papilloma formation initially paralleled that of the FVB littermates but the benign tumor burden peaked 9 weeks earlier than that of the controls. This premature peak was followed by a precipitous decline in papilloma burden that coincided with the early conversion of papillomas to carcinomas in the ProA mice. The observed decrease in carcinoma latency (6 weeks; $p = 0.02$), a 2.5 fold higher conversion frequency at 30 weeks post-initiation, and an increase in carcinoma size ($p = 0.03$) suggest that the pro-domain can act to enhance the transition of a benign papilloma to a malignant tumor in this model of squamous cell tumorigenesis. We conclude that the N-terminal pro-domain of IL-1α can cooperate with mutated H-ras to promote the outgrowth of initiated epithelial cells. A thorough understanding of the determining factors and pathways regulating the contribution of pro-IL-1α to tumor growth may provide a target for cutaneous tumor intervention.

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ENDOTHELIAL MONOCYTE-ACTIVATING POLYPEPTIDE–II (EMAP-II) INDUCES APOPTOSIS IN LYMPHOCYTES

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The novel pro-inflammatory cytokine EMAP-II (endothelial monocyte-activating polypeptide II), first found in tumour cell supernatants, is closely related or identical to the p43 auxiliary component of the multisynthase complex, which is involved in protein synthesis. *In vitro,* EMAP-II induces procoagulant activity on the surface of endothelial cells, increased expression of E-and P-selectins and TNF receptor-1, and is chemotactic for monocytes and neutrophils. The role of EMAP-II in tumours is not understood. We hypothesized that EMAP-II regulates activity of immune effector cells within neoplastic tissues, and investigated its effects on lymphocytes. EMAP-II caused a dose-dependent inhibition of proliferation, and apoptosis, in mitogen-activated PBMC and in Jurkat T-cells. Co-culture with HT29 or DLD-1 colorectal adenocarcinoma cells, or media conditioned by these cells, induced apoptosis in Jurkat cells, which could be partially reversed by addition of polyclonal antibodies against EMAP-II. EMAP-induced apoptosis, measured by annexin-V binding, was accompanied by caspase 8 activation. Our data suggest that EMAP-II released by, or expressed on the cell surface of tumour cells may be cytotoxic to lymphocytes, perhaps via a death receptor mechanism, and constitutes a component of a novel immunosuppressive pathway in solid tumors.

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EVALUATION OF THE EFFECTIVITY OF LYMPHOMA EL-4 CONTAMINATED BONE MARROW PURGING USING IL-2 INDUCED KILLER CELLS IN C57BL/6 MICE

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The purging of the autograft improves an efficacy of autologous bone marrow transplantation in leukemia and lymphoma. It was shown that stimulation of splenocytes with poor concentration of IL-2 and syngeneic lymphoma cells (SLC) turns splenocytes into killer cells (KC) with specific activity against SLC. We supposed that such KC could be effective in bone marrow purging. KC were generated by stimulation of C57Bl/6 mouse splenocytes with 100 IU/ml human lymphocyte IL-2 and irradiated SLC as stimulator. KC were washed and incubated during 6 hours with: 1) syngeneic EL-4 lymphoma contaminated bone marrow cells (SLCBMC); 2) intact bone marrow cells (BMC). KC/SLC ratio was 100/1. SLC cells were checked for proliferation capacity in vitro and in vivo. Lethal irradiated C57Bl/6 mice were inoculated i.v. with KC + BMC or KC + SLCBMC mixtures (1-2x10^6 bone marrow cells per mouse). From 80 to 100% of mice inoculated with KC + SLCBMC survived on day 35. 100% of mice died within 16-21 days after inoculating: SLCBMC alone or SLCBMC treated with intact syngeneic splenocytes. The results of the present study indicate that purging of bone marrow with syngeneic KC are highly effective in removal of contaminated SLC.

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CCL5 AND CCL2 in BREAST CANCER PROGRESSION: ACTIVITY AND REGULATION BY INTRINSIC AND MICROENVIRONMENTAL FACTORS

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Advanced breast carcinoma is highly correlated with elevated levels of CCL5 (RANTES) and CCL2 (MCP-1), supporting the malignant process by inducing the infiltration of monocytes to breast tumors. Our results suggest that the two chemokines mediate a pro-malignant interplay between the tumor cells and tumor-associated macrophages (TAM). CCL5 and CCL2 promote the expression of pro-malignant properties in monocytic cells (increased MMP and TNFα expression), while monocytic cell-derived products elevate the expression of tumor-supporting functions by the tumor cells (increased MMP and CCL5/CCL2 secretion). Using mammary adenocarcinoma cells differing in their malignancy phenotype, we demonstrated the concordant high expression of CCL2, IL-6 and MMP in highly tumorigenic mammary adenocarcinoma cells. By taking the clonal approach, we demonstrated that the three pro-malignancy factors are not co-regulated by a common intrinsic tumor-derived factor. Rather, TNFα, a TAM-derived pro-malignant microenvironmental factor in breast carcinoma, was shown to be a "master regulator" of CCL2, IL-6 and MMP expression. Our findings also demonstrate that the mammary tumor cell population is composed of a heterogeneous assortment of clones whose individual characteristics are averaged in the whole population. Furthermore, a highly malignant phenotype was shown to be determined, inter-alia, by a combinatorial effect of several pro- and anti-malignancy factors secreted by these cells.

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MOLECULAR ADJUVANTS FOR CANCER VACCINES: FROM BENCH TO BEDSIDE, WITH A STOP IN THE KENNEL

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The development of successful cancer vaccines is contingent on the ability to induce effective anti-tumor immunity against self-antigens that do not typically elicit immunity. We are working on strategies to overcome immunologic tolerance/ignorance to cancer through the use of gene products closely related to self-antigens, including homologous xenogeneic DNA. The potency of DNA vaccines can be further enhanced by adding DNA encoding cytokine genes. We have shown in preclinical mouse models that GM-CSF DNA induces an inflammatory response with production of IL-1β, IL-6, TNFα, CCL2, CCL3 and CCL5, and recruitment of PMNs and dendritic cells. In addition, GM-CSF DNA enhances antibody responses and tumor protection induced by immunizing C57BL/6 mice with human TYRP1 DNA. We have initiated a three-armed study in companion animals (dogs) with melanoma. Dogs are randomized to human GM-CSF DNA alone (3 dose levels), or a fixed dose of mouse tyrosinase DNA with or without h-GM-CSF DNA. Based on our data from mouse models, our expectation is that GM-CSF DNA will be a safe, convenient and effective adjuvant. The results will impact the design of future human clinical trials of anti-tumor vaccines, in which we plan to combine DNA vaccines with molecular adjuvants such as GM-CSF DNA.

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INHIBITION OF PROPROTEIN CONVERTASES ATTENUATES THE INDUCTION OF LIVER CYTOKINE, ADHESION MOLECULES AND METASTASIS BY COLON CARCINOMA CELLS IN MICE

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The proprotein convertases (PCs) are proteases directly responsible for the activation of several protein precursors implicated in the malignant phenotypes of tumor cells and tumor progression. Previously we reported that metastatic tumor cells entering the hepatic circulation rapidly induce a cytokine (Il-1 α and TNF-α) cascade leading to adhesion molecule (E-selectin, ICAM-1, VCAM-1) induction and subsequently liver metastasis (Khatib et al. Cancer Res, 1999, 59:1356-61). Here, we investigate the effect of the PC blockade on colon cancer cell induced-cytokine, adhesion molecules and colorectal liver metastasis. Using the general PC inhibitor α1-PDX and human (HT-29) and murine (CT-26) colon cancer cells, we find that stable transfection of HT-29 and CT-26 with α1-PDX results in a significant decrease of liver metastasis after their inoculation in the hepatic circulation into nude mice. Analysis of livers derived from HT-29/PDX or CT-26/PDX-injected mice, show low basal levels of cytokine and adhesion molecules mRNA as compared to those derived from HT-29 or CT-26-injected mice. In parallel, incubation of HUVEC cells with TNF-α or medium derived from HT29 or CT-26 cells significantly increases the adhesion of tumor cells to HUVEC as compared to media derived from HT-29/PDX or CT-26/PDX. This incremental increase in adhesion is dependent on the level of the adhesion molecule E-selectin expressed by HUVEC cells, as assessed by a neutralizing anti-E-selectin antibody. These results demonstrate the importance of the PCs in regulating molecules implicated in the early events of liver metastasis, thus suggesting that inhibition of PCs may provide a potentially useful strategy for the prevention of colorectal liver metastasis.

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INHIBITION OF B16 MELANOMA BY AS101 VIA RAS DEPENDENT DOWNREGULATION OF IL-10

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Malignant melanoma cells express different growth factors and cytokines in respective stages of tumor progression, which by autocrine and paracrine effects enable them to grow autonomously. AS101 (ammonium trichloro (dioxoethyine–0-0')tellurate) was previously found to exert antitumoral effects in various tumoral models. Its previous immunomodulating properties had been attributed to direct inhibition of IL-10 production at both mRNA and protein levels. In the present study we show that B-16 Melanoma cells constitutively secrete IL-10 in an autocrine manner. Exogenous addition of IL-10 to Melanoma cell cultures significantly augmented clonogenicity of treated cells in a dose dependent manner. We show that substantial amounts of IL-10 are secreted by B-16 Melanoma cells in a dose and time dependent manner. Moreover, IL-10 production is significantly inhibited by coincubation of the cells with increasing doses of AS101, which in parallel suppress B-16 Melanoma clonogenicity. Exogenous addition of IL-10 to AS101-treated cells abolished the inhibitory effect of AS101 on B-16 Melanoma growth, suggesting that this property of AS101 is exerted via down– regulation of IL-10. Furthermore, the inhibition of IL-10 by AS101 was ras dependent since coincubation of the cells with AS101 and Farnesyl transferase inhibitor significantly abolished this inhibitory property of AS101..

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CELLULAR RESPONSES TO THE TYROSINE KINASE FUSION PROTEIN TEL/PDGFbetaR ARE MODULATED IN THE PRESENCE OF CYTOKINE

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The oncogenic TEL/PDGFbetaR fusion protein recurrently occurs in patients with chronic myelomonocytic leukemia (CMML), due to a t(5;12)(q33; p13) chromosomal translocation. We have used a Tet-regulated system to express TEL/PDGFbetaR in IL-3-dependent BaF/3 cells to investigate the coupling of molecular signalling events activated by this oncogene to functional responses. Clones induced to express TEL/PDGFbetaR demonstrate increased cell survival upon withdrawal of IL-3, and constitutive activation of PKB, STAT5, ERK1/2, JNK1/2 and p38 MAPK pathways. Inducible expression of TEL/PDGFbetaR failed to transform clones to factor-independence, whereas prolonged constitutive expression did, albeit at a low frequency, suggesting secondary events are required for transformation. Surprisingly, IL-3-dependent growth was dramatically reduced and apoptosis increased in cells expressing TEL/PDGFbetaR. TEL/PDGFbetaR augmented IL-3-induced activation of PKB, STAT5, ERK1/2, p38 and JNK1/2. Reducing the activity of PI3K and p38 did not reverse this inhibition of IL-3-driven proliferation. However, inhibition of either the ERK or JNK cascades partially reversed these inhibitory effects. Our study suggests that the combination of TEL/PDGFbetaR and IL-3-induced signals activate apoptosis through ERK and JNK dependent pathways. Given that the bone marrow environment exposes haemopoietic cells to a variety of cytokines, these results have important implications for cellular responses in the pathogenesis of CMML.

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MIC-1 SERUM LEVEL AND GENOTYPE: ASSOCIATIONS WITH PROGRESS AND PROGNOSIS OF COLORECTAL CARCINOMA

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Epithelial cells are highly proliferative and prone to the development of neoplasia. These cells are subject to important regulatory influences, prominent amongst which are the TGF-b cytokines. Macrophage inhibitory cytokine-1 (MIC-1) is a divergent member of the TGF-b superfamily. Two allelic variants of MIC-1 were identified differing by one amino acid termed H and D. An ELISA assay has been developed that allows the detection of MIC-1 in serum and this has allowed us to asses it role in CRC.
We determined the serum MIC-1 level, MIC-1 genotype or both in three separate cohorts of subjects who were normal, had adenomatous polyps or CRC. The normal range for serum MIC-1 level was 200-1150 pg/ml. There was an incremental increase in serum MIC-1 levels between normals, those with adenomatous polyps and again in those with CRC. Serum MIC-1 level was positively related to Duke's stage of disease and the presence of metastasis. There were significant allelic effects on disease course. CRC patients with a D allele were more likely to present with metastasis. Those subjects who were homozygous H for MIC-1 tended to relapse latter than those subjects with a D allele, but also died sooner. This data suggests that MIC-1 has a role in human tumour cell regulation *in vivo* and that measurement and determination of genotype of MIC-1 may have clinical as well as therapeutic utility.

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MACROPHAGE INHIBITORY CYTOKINE 1 REDUCES CELL ADHESION AND INDUCES APOPTOSIS IN PROSTATE CANCER CELLS

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Macrophage inhibitory cytokine 1 (MIC-1), a divergent member of transforming growth factor b superfamily, is linked to the pathogenesis of cancer. In order to delineate possible roles for MIC-1 in prostate cancer we have used a number of prostate epithelial cell lines including PZ-HPV-7, DU-145, PC-3 and LNCaP cells. We have investigated both factors regulating the production of MIC-1 protein by these cells and also determined some of the effects of MIC-1 on them. While PZ-HPV-7 and DU-145 produced no MIC-1 protein, PC-3 and LNCaP cells secreted MIC-1 protein at high levels. The secretion of MIC-1 in LNCaP cells was modulated by both androgen and estrogen. While neither MIC-1 nor anti-MIC-1 antibody had any effect on proliferation of the epithelial cells, MIC-1 induced changes in DU145 cells. These cells became flattened and more spread and this was accompanied by reduced intercellular actin filaments and intercellular junctions. The DU-145 cells then detached from their substrate and underwent caspase-dependent apoptosis. In order to define some of the genes responsible for these changes, cDNA microarrays followed by confirmatory RT-PCR was used to analyze differential gene expression induced by MIC-1. The anti-apoptotic gene metallothionein 1E and cell adhesion genes RhoE and catenein δ1 were down-regulated by more than 2 fold by MIC-1, suggesting that they were at least in part responsible for the observed changes in the behavior of DU145 cells. These findings suggest that whilst MIC-1 has no effect on cell proliferation, it reduces cell adhesion and consequently induces cell detachment. It is likely that caspase-dependent apoptosis is secondary to loss of cell adhesion and may suggest a role for MIC-1 in tumor dissemination *in vivo*.

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THERAPEUTIC OPPORTUNITIES FOR IL-21

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The IL-2 family of cytokine's plays a pivotal role in adaptive immune responses. Although they function through a receptor complex containing the shared gamma common subunit, these cytokines regulate distinct aspects of humoral and cell-mediated immunity and often compliment or antagonize each other's activities. The most recently identified member of this interleukin family is IL-21. Initially, IL-21 was shown to modify the proliferation and differentiation of T-cells, B-cells, and NK-cells *in vitro*. More recent efforts have uncovered how IL-21 omay function *in vivo*. Using a variety of mouse models, we have learned that injection of recombinant mouse IL-21 can mediate a potent anti-tumor response that is accompanied by the marked expansion of tumor-specific CTL. Significantly, systemic delivery of IL-21 is non-toxic in mice relative to other cytokine family members. Thus, IL-21 demonstrates therapeutic potential in the context of cancer immunotherapy

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SELECTIVE EXPRESSION AND FUNCTION OF CHEMOKINE RECEPTORS IN HUMAN PANCREATIC ADENOCARCINOMA

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In this study we have investigated the expression patterns of a number of chemokine receptors in pancreatic adenocarcinoma cell lines and in primary pancreatic tumors, in order to understand if these receptors and their ligand chemokines are involved in tumor dissemination. We have extensively analyzed by Real Time PCR and surface phenotype a large panel of chemokine receptors on twelve pancreatic tumor cell lines. The chemokine receptor CXCR4 was expressed in half of cell lines (6/12). CCR7 was significantly expressed in four cell lines and CCR6 only in two of them, with individual heterogeneity. CCR2 and CCR5 were not significantly expressed. Primary tumor cells isolated from surgical specimens (> 95% cytokeratine-7 positive) generally expressed higher levels of chemokine receptors compared to cell lines and normal pancreatic ducts. Flow cytometric analysis with specific mAb generally confirmed these results at the protein level. The functional activity of chemokine receptors expressed by tumoural cells was tested *in vitro*. Two CXCR4-positive cell lines and one CCR7 positive cell line dose-dependently migrated in response to appropriate ligands in classical chemotaxis assays as well as in trans-endothelial migration assay. CXCL12 was also found to rescue tumor cells from serum deprivation-induced apoptosis. Overall these results demonstrate that pancreatic tumor cells express selected and functional chemokine receptors which may have a role in the survival and dissemination of pancreatic cancer.

CHEMOKINES

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THE CXCR3 LIGANDS IP-10, ITAC AND MIG STIMULATE PI3K-DEPENDENT SIGNALLING EVENTS IN INTESTINAL MYOFIBROBLASTS

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Wound healing is a complex response that involves recruitment of inflammatory cells and the deposition of extracellular matrix. Myofibroblasts play a pivotal role in this process and can both express chemokines and be targets of the action of chemokines. The constitutive and regulated production of the IFN-γ inducible chemokines, IP-10, Mig and ITAC by human intestinal epithelium and the expression of their cognate receptor CXCR3, by intestinal myofibroblasts suggest that interactions between these cells can play a role in modulating physiologic and pathologic mucosal inflammation. Here we show, using primary intestinal myofibroblasts that CXCR3 ligation with IP-10, I-TAC or MIG leads to elevation of intracellular calcium levels. The CXCR3 ligands also stimulated phosphorylation of ERK which was inhibited by the MEK inhibitor PD98059 and the PI3K inhibitor LY294002. These chemokines also stimulate, a rapid and transient phosphorylation of the PI3K effector PKB as well as the MAPK family member p38. Phosphorylation of ERK, p38 and PKB are inhibited by pre-treatment with the Gαi inhibitor pertussis toxin. Finally *in vitro* assays indicate activation of the class II PI3K α and β isoforms in response to IP-10 and MIG. Ongoing work will further define the functional importance of these signalling pathways in myofibroblasts.

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CCL5-CCR5 INTERACTIONS INDUCE APOPTOSIS IN T CELLS: REQUIREMENT FOR GLYCOSAMINOGLYCAN BINDING AND CCL5 AGGREGATION

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The chemokine CCL5 (RANTES) and its cognate receptor, CCR5, have been implicated in antigen-independent T cell activation. In PM1 cells, a CCR5 expressing human T cell line, μM concentrations of CCL5 induce apoptosis via activation induced cell death. We provide evidence that CCL5 inducible apoptosis, although mediated by CCR5, is dependent on cell surface GAG binding. CCL5 binding to glycosaminoglycans (GAGs) may serve to induce chemokine aggregation, present chemokine to its associated receptor and facilitate receptor interactions. Studies with two aggregation-mutant forms of CCL5, E66S and E26A, show that tetramers are the minimal higher order forms required to induce cell death. Our data also suggest that CCL5 activation of CCR5 that leads to apoptosis may involve other cell surface receptor components, such as CD3. Specifically, in MOLT-4 T cells that express CCR5 and not CD3, CCL5-CCR5 mediated apoptosis is attenuated. We have extended these studies to examine the effects of treating primary human CD3+, CCR5+ T cells with μM doses of CCL5. These data will be discussed in the context of CCL5-CCR5 interactions regulating activation induced cell death, thereby determining T cell fate.

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PI3K-DEPENDENT SIGNALLING EVENTS ARE NOT SUFFICIENT FOR CCR4-STIMULATED T LYMPHOCYTE CHEMOTAXIS

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A hallmark of inflammatory responses is the migration of leukocytes to the inflammatory lesion in response to chemokines/chemoattractants. Several studies have shown that polarised activation of class 1 phosphoinositide 3-kinase (PI3K) at the leading edge of migrating cells is a crucial and early event in the detection of a chemoattractant gradient. Here, we provide evidence that cell migration stimulated by the chemokine receptor CCR4 can occur in the absence of detectable PI3K activation. CCR4 is predominantly expressed on T cells of the Th2 lineage and may play a pivotal role in Th2-associated diseases. The two known CCR4 ligands, MDC (CCL22) and TARC (CCL17) stimulate robust cell migration of polarised Th2 cells and the CEM T cell line. In addition, both CCR4 ligands stimulate PI3K activation as assessed by accumulation of $PI(3,4,5)P_3$ and phosphorylation of the PI3K effector PKB/Akt. Both events are inhibited by pre-treatment with the Gαi inhibitor pertussis toxin as well as the PI3K inhibitors LY294002 and wortmannin. Surprisingly, CCR4-mediated chemotaxis is *insensitive* to these PI3K inhibitors. This data casts doubt on the importance of PI3K activation for migratory responses and suggests other signalling events can sustain cell migration in the absence of any PI3K signal.

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REGULATION OF CXCR4-STIMULATED PI3K-DEPENDENT SIGNALLING EVENTS BY THE LIPID PHOSPHATASE SHIP

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The SH2 domain-containing inositol polyphosphate 5-phosphatase (SHIP) is known to play an important role in the negative regulation by FcγRIIb of PI3K-dependent signalling cascades activated by the B cell antigen receptor (BCR) as well as several tyrosine-kinase coupled cytokine receptors. However, to date the role of SHIP in the regulation of PI3K-dependent signals elicited by G-protein coupled receptors such as chemokine receptors have not been investigated. In this study, we report that ligation of CXCR4 by CXCL12 has no effect on the tyrosine phosphorylation of SHIP in the murine B cell lymphoma A20. We investigated the effect of FcγRIIB ligation on CXCL12 mediated chemotaxis, in addition to the PI3K-dependent phosphorylation of PKB and ERK1/2. We have also utilised a constitutively active membrane-localised SHIP mutant expressed in the Jurkat leukaemic T cell line, which do not normally express SHIP, to look at the effect of this mutant on CXCL12 stimulated PI3K-dependent signalling events such as PKB phosphorylation and $PI(3,4,5)P_3$ accumulation. Experiments have revealed that CXCL12 stimulated PKB phosphorylation is severely abrogated in the presence of this SHIP mutant. Thus, it appears that activation of SHIP can impinge on PI3K-dependent signalling pathways activated by G-protein coupled receptors

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CXCR1 AND CXCR2: REGULATION OF RECYCLING AND OF CXCL8-INDUCED MIGRATORY RESPONSES

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CXCR1 and CXCR2 mediate migratory activities in response to CXCL8 (IL-8) and other ELR$^+$-CXC chemokines. Our studies indicate that both receptors undergo recycling through phosphatydilinositol- and actin-dependent processes. Receptors mutated in carboxyl terminus serine/threonine residues are recycled normally. However, such receptors are not recycled through phosphatidylinositol-, actin- or microtubule-dependent pathways. Insight into the regulation of ELR$^+$-CXC chemokine-induced migration was gained by the observation that both Focal Adhesion Kinase (FAK) and Pyk2 are phosphorylated by exposing the cells to CXCL8. Under conditions of migratory activation (e.g. 10-50 ng/ml CXCL8), FAK is phosphorylated in a $G_{\alpha i}$-dependent manner. FAK activity was shown to be directly required for chemotaxis. However, in conditions of migration desensitization (1000 ng/ml CXCL8), further promotion in FAK phosphorylation levels was observed (in 4 out of its 6 phosphorylation sites), indicating that FAK is differently regulated under migratory-attenuating *vs* migratory-activating conditions. Further analysis demonstrated that FAK phosphorylation is partially dependent on Src kinases, however the role of these kinases differs for CXCR1 *vs* CXCR2 stimulation. In addition, our study has indicated that CXCL8 stimulation results not only in FAK phosphorylation but also in its redistribution to membrane regions that form definite contact areas with the substratum.

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INDUCTION OF CXCL8 in HUMAN PHARYNGEAL CELLS BY PNEUMOLYSIN AND CHOLINE-BINDING PROTEIN A

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In murine studies several pneumococcal proteins including pneumolysin (ply) and choline-binding protein A (CbpA) are important virulence factors. Used as vaccines they can prevent infection and/or carriage.
CXCL8 is a potent inflammatory mediator and immunemodulator.We have investigated the effect of pneumococcal proteins on CXCL8 production in the human upper respiratory tract epithelial cell line (Detroit 562). Concentrated culture supernatant (CCS) from a type 2 pneumococcus strain (D39) was incubated with Detroit cells and CXCL8 production measured by ELISA.
Secretion of CXCL8 was significantly increased in CCS-stimulated cells when compared to controls ($p < 0.001$). Pre-incubation of CCS with proteinase K nearly abolished CXCL8 production, suggesting that the induction of this chemokine is due to secreted pneumococcal protein. SDS-PAGE and Western blotting showed that the CCS contained secreted proteins including Ply and CbpA. Stimulation of Detroit cells with recombinant Ply and CbpA (8-500 ng/mL and 0.5-10 ig/mL, respectively) elicited production of CXCL8 in a dose-dependent manner.
Ply and CbpA induce significant CXCL8 production by upper respiratory airway epithelial cells. These data may inform design of pneumococcal vaccines.

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REPERTAXIN IS A NEW POTENT AND SELECTIVE SMALL ORGANIC INHIBITOR OF INTERLEUKIN-8 (CXCL8) WITH A UNIQUE MODE OF ACTION

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Interleukin-8 (CXCL8) is known to play a key role in directing leukocyte trafficking in inflammatory sites. Inhibition of CXCL8 biological activities may represent an attracting therapeutic target in several inflammatory diseases. We have recently identified repertaxin as a new potent and selective small organic inhibitor of CXCL8. Repertaxin selectively inhibits CXCL8 induced neutrophil chemotaxis *in vitro* and prevents neutrophil infiltration and tissue damage in ischemia/reperfusion animal models. The aim of this study was to investigate the mechanism of action of repertaxin. Our results show that repertaxin does not compete with CXCL8 receptor binding on human neutrophils or CXCR1/CXCR2 bearing cells, suggesting that repertaxin is not a receptor antagonist. Repertaxin inhibits signal transduction events downstream to CXCL8 receptor binding, including G-protein activation, Pyk2 tyrosine phosphorylation and intracellular calcium increase. Photolabelling experiments show that repertaxin binds to CXCR1 receptor. These results, along with modelling and CXCR1 mutation analysis, suggest that repertaxin and related compounds act as non-competitive allosteric blockers by interacting with transmembrane domains of CXCL8 receptors.

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THE sIL-6R IN ARTHRITIS: BLOCKADE OF DISEASE PROGRESSION BY s-gp130

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Recent clinical trials point to IL-6 as a potential target for the treatment of rheumatoid arthritis. Studies in IL-6-deficient (IL-6-/-) mice highlight that IL-6 contributes to arthritis progression, however the molecular mechanism controlling its activity *in vivo* within the rheumatoid synovium remains unclear. Using an experimental arthritis model in IL-6-/- mice we have established a critical role for the sIL-6R in joint inflammation. Although intra-articular administration of IL-6 itself was insufficient to reconstitute arthritis, a sIL-6R-IL-6 fusion protein (HYPER-IL-6) restored disease activity. Histopathology of joint sections demonstrated that HYPER-IL-6 increased arthritis severity and controlled intra-synovial mononuclear leukocyte recruitment through the CC-chemokine CCL2. Activation of synovial fibroblasts by sIL-6R and IL-6 emphasized that these cells may represent the source of CCL2. Comparable FACS analysis of IL-6R expression on leukocytes from blood and synovial fluids obtained from rheumatoid arthritis patients showed that cognate IL-6R levels are lower on synovial leukocytes suggesting that transmigration promotes sIL-6R release. Indeed, neutrophils stimulated with chemotactic agents induced sIL-6R shedding *in vitro*. Blockade of sIL-6R signaling in wild type mice using sgp130 ameliorated disease by suppressing CCL2-driven leukocyte recruitment, synovial hyperplasia and overall joint erosion. Consequently, soluble IL-6R–mediated signaling represents a promising therapeutic target for the treatment of rheumatoid arthritis.

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CLONING AND EXPRESSION OF A DIFFERENTIALLY SPLICED ISOFORM OF SOLUBLE-gp130 AND ITS ROLE IN INFLAMMATORY DISEASE

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A key feature in the regulation of IL-6 responses has been the identification of a soluble interleukin-6 receptor (sIL-6R), which forms a complex with IL-6 capable of inducing a variety of inflammatory responses. These responses are thought to be counteracted by a soluble form of gp130 (sgp130). We have demonstrated that a single intra-articular injection of sgp130 significantly inhibits joint inflammation in an experimental murine model of arthritis in association with reduced CCL2-driven mononuclear cell synovial infiltration. Several studies have identified at least 3 different isoforms of sgp130 including 50- 90- and 110kDa forms, and the 110kDa sgp130 (DS-sgp130) was identified in a limited number of RA synovial fluids (11 ± 3.1 ng/ml). Of particular interest is a 50kDa protein (gp130-RAPS; Rheumatoid arthritis Antigenic Peptide-bearing Soluble form), which possesses a unique COOH-terminal sequence (NIASF). Rabbit antiserum specific for gp130-RAPS was raised against the unique NIASF motif and identified this isoform in synovial fluids from rheumatoid and osteoarthritic patients. To investigate the role of gp130-RAPS in arthritic disease, a soluble recombinant form of gp130-RAPS has been expressed in bacteria transformed with the pASK-IBA2 expression vector containing full-length gp130-RAPS cDNA derived from human synovial fibroblasts. Purification and characterization of this novel protein is currently being carried out.

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THE sIL-6R DIFFERENTIALLY REGULATES NEUTROPHIL-ACTIVATING CHEMOKINE EXPRESSION

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IL-6 signalling *via* its soluble receptor (sIL-6R) differentially regulates inflammatory CXC- and CC-chemokine expression and leukocyte apoptosis to coordinate a switch from neutrophil to mononuclear cell infiltration. Soluble IL-6R activities may however be influenced *in vivo* by two isoforms that are released through either differential mRNA splicing (DS) or proteolytic cleavage (PC) of the cognate IL-6R. Using human mesothelial cells and a murine model of peritoneal inflammation studies compared the ability of both isoforms to regulate neutrophil recruitment. In terms of neutrophil infiltration, DS- and PC-sIL-6R were comparable in their activities, however these studies emphasised that sIL-6R differentially controls neutrophil-activating CXC-chemokine expression. *In vitro*, stimulation of mesothelial cells with IL-6 and DS- or PC-sIL-6R showed no induction of CXCL1 and CXCL8, whereas both isoforms enhanced CXCL5 and CXCL6 expression. Moreover, when complexed with IL-6 both isoforms blocked the IL-1β-induced secretion of CXCL8. These findings were paralleled *in vivo*, were induction of peritoneal inflammation in IL-6-/- mice resulted in enhanced KC and MIP-2 (equivalent to CXCL1 and CXCL8), and reduced LIX (equivalent to CXCL5) levels. Reconstitution of IL-6 signaling in IL-6-/- mice with IL-6 and its soluble receptor isoforms restored chemokine expression and overall suppressed neutrophil infiltration. Thus, sIL-6R-mediated signaling primarily impedes neutrophil influx, but through induction of CXCL5 and CXCL6 may also regulate other neutrophil responses.

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IL-6 DEFICIENCY DISRUPTS T-CELL RECRUITMENT DURING ACUTE INFLAMMATION

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During acute inflammation IL-6 signalling through its soluble receptor (sIL-6R) facilitates the transition between neutrophil and mononuclear cell infiltration. In IL-6-deficiency this process is defective, and results in heightened neutrophil infiltration and impaired mononuclear cell recruitment. This pattern of recruitment is governed by sIL-6R, which differentially balances inflammatory chemokine expression and leukocyte apoptosis. Here we characterise IL-6 as a regulator of lymphocyte recruitment. Acute peritoneal inflammation was induced in wild-type (WT) and IL-6-deficient mice by administration of *S. epidermidis* cell-free supernatant (SES). At intervals (0-96 hours) the lymphocyte infiltrate was phenotyped by FACS using monoclonal antibodies against pan lymphocyte markers and the chemokine receptors CCR3, CCR4, CCR5, CXCR3 and CXCR5. Following 18-hours SES stimulation, T-cell recruitment significantly increased with peak infiltration occurring after 48-hours. Analysis of the T-cell infiltrate revealed a differential pattern of chemokine receptor expression, with optimal CXCR3 (24hrs), CCR5 (36hrs) and CCR3/CCR4 (48hrs) levels resulting at distinct stages during the inflammatory response. In IL-6-deficiency, lower levels of these receptors were detected on the T-cell infiltrate, which corresponded with temporal defects in peritoneal CCL2, CCL5, CXCL10 and CCL17 levels (CXCL13 expression and CXCR5 + B-cell recruitment was unaffected). Indeed sgp130 blockade of sIL-6R-mediated signalling in WT mice disrupted expression of these chemokines suggesting that sIL-6R-mediated signalling directs acquired immune responses during acute inflammation.

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IFNa ENHANCES THE CHEMOTAXIS OF MEMORY B CELLS IN DECREASING LIGAND-INDUCED CHEMOKINE RECEPTOR INTERNALIZATION

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We analyzed the effect of IFNα on the migration of human naive and memory B cells in response to MIP-3α/CCL20, SLC/MIP-3β/CCL19 and SDF-1/CXCL12. IFNα increased the specific chemotaxis of B cells in a dose-dependent manner. The effect was maximal with 2000 IU/ml of IFNα. The IFNα-induced increase in chemotaxis was first detected after 2 hrs, peaked after 24 hrs and decreased thereafter. At 24 hrs, the average IFNα-induced increase in chemokine-specific migration was equal to 20.6 ± 6.3% for MIP-3α (n = 8), 36.6 ± 14.4% for SLC (n = 15) and 14.6 ± 3.9% for SDF-1 (n = 5). The IFNα-induced increase in specific chemotaxis correlated with an increase in the percentage of memory B cells in the migrating population. With SLC, there were two times more memory B cells than naive B cells and with MIP-3α or SDF-1 there were five times more. Thus, IFNα preferentially impairs the chemotaxis of memory B cells. To exert its effect on B cell chemotaxis, IFNα reduces the ligand-induced receptor internalization by a PI3K-dependent mechanism whereas it increases the chemokine-induced phosphorylation of ERK2 by three to four fold. Thus, IFNα regulates the chemotaxis of human memory B cells by an unknown mechanism, possibly impairing β-arrestin trafficking.

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MICROBIAL TOLL-LIKE RECEPTOR LIGANDS DIFFERENTIALLY REGULATE CXCL8 AND CXCL10 EXPRESSION IN FIBROBLASTS AND PBMC: ENHANCED SYNOVIAL CHEMOKINE LEVELS IN SEPTIC ARTHRITIS

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The CXC chemokine interferon-γ-inducible protein-10 (IP-10/CXCL10) attracts activated T cells and natural killer cells. Compared to the toll-like receptor 3 (TLR3) ligand, double stranded (ds) RNA, IFN-γ is a superior IP-10 inducer on PBMC. The bacterial TLR4 and TLR2 ligands, LPS and peptidoglycan (PGN), inhibit IFN-γ- or dsRNA-dependent IP-10 production in PBMC, whereas IL-8/CXCL8 production was enhanced. In fibroblasts, IFN-γ induces moderate and dsRNA provokes strong IP-10 production. Furthermore, bacterial LPS and PGN synergize with IFN-γ to trigger IP-10 and IL-8 production in fibroblasts. The synergistic induction of IP-10 in fibroblasts is reflected by significantly enhanced IP-10 in synovial fluids of septic compared to osteoarthritis patients to reach on average higher levels than those of IL-8. Thus, IP-10 produced by activated connective tissue fibroblasts may attract CXCR3 expressing activated Th1 cells and natural killer cells, antagonize the CCR3 dependent attraction of Th2 lymphocytes and exert receptor-independent, defensin-like antibacterial activity.

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MÜLLER GLIAL CELLS FROM GUINEA PIG AND HUMAN RETINAS PRODUCE IL-8 AND EXPRESS CXCR1 AND CXCR2 RECEPTORS

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Interleukin-8 (IL-8) is a pro-inflammatory chemokine. Several diseases of the eye (e.g. uveitis, melanoma, PVR) are associated with increased levels of IL-8 in the vitreous. Various cell types, such as retinal pigment epithelium cells, fibroblasts or ciliary epithelial cells, are potential sources for the secreted IL-8. The aim of our study was to evaluate whether retinal glial cells are able to produce and secrete IL-8 and/or to express IL-8 receptors. We established primary cultures of isolated Müller cells from guinea pig and human retinas and used an immortalized human Müller cell line. Additionally, we evoked an inflammation in guinea pig eyes by application of LPS. The Müller cell cultures were prepared for immunocytochemistry, Western blotting and RT-PCR, and the supernatants were used for ELISA analysis. Ca^{2+} imaging was performed to test the activation of IL-8 receptors. The retinas from the infected eyes were used for immunocytochemistry and Western blotting.
IL-8 mRNA could be detected in human and guinea pig Müller cell cultures. IL-8 immunoreactivity was accompanied by GFAP immunoreactivity and was colocalized with other glial specific markers. ELISA analysis of the supernatants revealed that IL-8 was secreted from Müller cells into the culture medium. Immunoreactivity for CXCR1 and CXCR2 could be detected in human and guinea pig Müller glial cells. Western blot analysis revealed the detection of proteins with about 40 kDa by using CXCR1 and CXCR2 specific antibodies. The RT-PCR method confirmed the expression of CXCR1 and CXCR2 in the human Müller cell line. Application of recombinant IL-8 protein to the cultured Müller cells caused an increase in intracellular Ca^{2+} levels in subpopulations of Müller cells. Immunostainings for IL-8 and both IL-8 receptors revealed an expression in Müller cells in the LPS-treated eyes. It is concluded that Müller cells may participate in the inflammatory response of pathologically altered or injured eyes.

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CXCR5 CONTRIBUTES TO BUT IS NOT ESSENTIAL FOR HIGH ENDOTHELIAL VENULES IN LYMPHOID ORGAN DEVELOPMENT

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Knockout and transgenic studies of LTα:TNFRI, LTαβ:LTβR and CXCL13:CXCR5 ligand:receptor pairs revealed important roles LN development. Transgenic mice that express LT under control of the rat insulin promoter II (RIP) develop ectopic lymphoid structures resembling LNs and serve as a model of lymphoid organogenesis. LN function depends, in part, on high endothelial venules (HEV) expressing peripheral node addressin (PNAd), an adhesion molecule important for L-selectin$^+$ cell entrance into lymphoid tissue. We have previously shown that LTαβ contributes to HEV development and optimal PNAd expression through induction of a HEV-restricted sulfotransferase (HEC-6ST). To further investigate the interplay between CXCR5, LTα, and LTαβ in LN development, with particular attention to HEV phenotype, we analyzed lymphoid organs in C57BL/6, CXCR5$^{-/-}$, LTb$^{-/-}$, and RIPLTa.CXCR5$^{-/-}$ mice. CXCR5$^{-/-}$ and LTβ$^{-/-}$ mice lack peripheral LN (PLN) and retain mesenteric and cervical LNs (MLN and CLN). CXCR5$^{-/-}$ MLN disorganized T and B cell compartments and fewer FDC networks. Both CXCR5$^{-/-}$ and LTβ$^{-/-}$ MLN exhibited HEV with abnormal patterns of PNAd expression displaying reduced levels of HEC-6ST expression and a decrease in L-selectin$^+$ cells; 50% reduction in CXCR5$^{-/-}$mice and 30% reduction in LTb$^{-/-}$ mice. Interestingly, RIPLTa CXCR5$^{-/-}$ mice exhibited a partial restoration of PLNs with HEV expressing HEC-6ST and PNAd. These studies highlight a role for CXCR5 in HEV development but indicate that constitutive LTa expression can partially compensate for its absence in LN development.
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GATA-BINDING PROTEIN 2 IS INVOLVED IN INHIBITORY EFFECT OF CKβ8-1 ON COLONY FORMATION OF HEMATOPOIETIC STEM CELLS

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A C6β-chemokines, CKβ8-1, suppressed colony formation of multipotential granulocyte erythroid megakaryocyte macrophage (CFU-GEMM), granulocyte-macrophage (CFU-GM), and erythroid (BFU-E) from cells of cord blood (CB). To investigate genes in CB cells regulated by CKβ8-1, membrane arrays containing approximately 9,000 human genes were hybridized to labeled cDNA populations from the cultured CB cells treated with and without CKβ8-1. We observed 12 genes which were significantly regulated. By using real time PCR analysis, we demonstrated that gene encoding GATA-binding protein 2 was not expressed in CKβ8-1-treated cells while they were highly expressed in control cells. Murine bone marrow cells were transfected with siRNA against GATA-binding protein 2. Colony forming assay of these transfected hematopoietic stem cell demonstrated that GATA-binding protein 2 is involved in control of hematopoiesis. These results indicated that inhibitory effect of CKβ8-1 on colony formation of CB cells may be mediated thru GATA-binding protein 2.

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REGULATION OF THE CHEMOKINE RECEPTOR CXCR4 BY HYPOXIA

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Cell adaptation to hypoxia requires activation of transcriptional programs that coordinate expression of genes involved in oxigen delivery (via angiogenesi) and metabolic adaptation (via glycolisis). During migration and invasion of normal and phatological tissues, cells may encounter different oxygen levels, due poor or altered vascularization, and recent evidence have suggested that chemotaxis is a cell function which may be affected by oxygen availability.
Here we describe that oxygen avaibility is a determinant parameter in the setting of chemotactic responsiveness to the Stromal-Derived Factor 1 (SDF-1, CXCL12). Low oxygen concentration induces both mRNA and surface expression of the CXCL12 receptor CXCR4, in different cell types (monocytes, monocyte-derived macrophages, tumor associated macrophages, endothelial cells and cancer cells), which is paralleled by increased chemotactic responsiveness to its specific ligand. CXCR4 induction by hypoxia is dependent on both activation of the hypoxia-inducible factor 1 (HIF-1α) and transcript stabilization.
Our data suggest that hypoxia/HIF-1/CXCR4 pathway may be crucial in physiological and pathophysiological responses to hypoxia.

EFFECTORS MECHANISMS

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INTERLEUKINS, ADHESION MOLECULES AND NEUTROPHIL ACTIVATION IN ARDS

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Development of acute respiratory distress syndrome (ARDS) often determines of pure outcome of severe acute pancreatitis during the first week of the disease onset. The goal of this investigation was to determine the role of cytokines and adhesion molecules on the ARDS development.
The levels of interleukins 1β, 8, 12, and 18, levels of soluble adhesion molecules (sE-selectin, sICAM-1, and sVCAM-1), myeloperoxidase and reactive oxygen species (hydrogen peroxide) were studied in 14 patients with confirmed ARDS and severe pancreatitis. The 24 patients with necrotizing pancreatitis, but without ARDS, compiled the control group.
Already at the first hours of the disease onset the highest levels of all interleukins, myeloperoxidase and hydrogen peroxide serum levels were noted in patients, which in subsequent had ARDS in compared with control group ($p < 0,05$). During the first 72 hours the elevation of cytokines, especially IL-1β IL-8, and IL-18 were correlate with the increasing of severity of respiratory failure. The increased levels of myeloperoxidase and hydrogen peroxide pointed on neutrophils activation, for which IL-8 and IL-18 are the powerful hemoatractants.The levels of sE-selectin and sICAM-1 increasing too, but levels of sVCAM-1 practically did not changed. Infusion of pentoxifylline in the daily dose 400-600 mg led to significant decrease of interleukins and adhesion molecules and corresponding the severity of ARDS.
Thus, the interleukins and adhesion molecules play an important role in the ARDS development in patients with acute pancreatitis. The pentoxifylline infusion, which has anticytokines properties, is necessary component of the intensive care management of acute pancreatitis.

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SIGNALING THROUGH 4-1BB (CD137) NEUTRALIZES THE SUPPRESSIVE ACTIVITIES OF ACTIVATED $CD4^+CD25^+$ REGULATORY T CELLS

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4-1BB (CD137) is a T cell costimulatory molecule invovled in the activation and survival of CD4, CD8, and NK cells. We tested the role of 4-1BB-4-1BBL pathway in the modulation of suppressive function of $CD4^+CD25^+$ regulatory T cells. In spite of functional 4-1BB expression on $CD4^+CD25^+$ regulatory T cells, the contribution of 4-1BB to the proliferation of the regulatory T cells is minimal. Signaling through 4-1BB receptor, however, effectively ($p < 0.05$) neutralizes the suppressive function of $CD4^+CD25^+$ regulatory T cells in both *in vitro* and *in vivo*. Such a desuppressive activity of 4-1BB is much more potent in activated $CD4^+CD25^+$ regulatory T cells.

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GRANZYME B AND IFN-γ PRODUCTION BY EFFECTOR CELLS IN CELL-MEDIATED CYTOTOXICITY

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One of the major mechanisms of cell-mediated cytotoxicity involves the release of cytoplasmic granules by effector cells. These granules contain, among others, the cytolytic protein Granzyme B (GrB) that is thought to be critical in the induction of target cell apoptosis. Using a GrB ELISPOT assay we were able to detect GrB production by NK and CTL as early as in 10 min after initial contact with target cells. Using flow cytometry we demonstrated rapid transfer of GrB from effectors into target cells and phoshatidylserine redistribution on the target cell membrane. Both events were perforin and calcium dependent. When the GrB ELISPOT assay was directly compared to the IFN-γ ELISPOT assay, excellent correlation between these assays was shown, though significant IFN-γ production was detectable later than GrB production. After $CD8^+$ cells were removed from anti-FMP CTL, both IFN-γ and GrB production was practically abrogated. Both ELISPOT assays have shown excellent correlation with ^{51}Cr release assay, but were significantly faster and more sensitive. The data presented suggest that target cell recognition can trigger rapid release of both GrB and IFN-γ, however it is not clear at present if they are produced by the same cells.

GENE REGULATION

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INDUCIBLE EXPRESSION OF FRA-1 IS REQUIRED FOR SUSTAINED INTERLEUKIN (IL)-1-INDUCED IL-8 TRANSCRIPTION

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IL-1 treatment of human KB epithelial cells induces a very rapid and strong (>100-fold) induction of IL-8 protein, whereas EGF induces only a 10-fold increase in IL-8 synthesis. To elucidate the molecular mechanism for this quantitative difference in IL-8 expression, we analysed the composition of IL-1 and EGF-induced protein complexes at the endogenous IL-8 promoter. IL-1 activates IL-8 transcription potently through both, NF-κB and AP-1 bindings sites of the IL-8 promoter, whereas EGF utilizes mainly the AP-1 binding site. As assessed by chromatin-immunoprecipitation IL-1 rapidly and potently induces recruitment of RNA polymerase II and p65 NF-κB to the endogenous IL-8 promoter, whereas EGF only weakly induces RNA Pol II binding and p65 recruitment. Further experiments were performed to reveal how the AP-1 site contributes to IL-8 expression. By both, RNAi and cell-permeable peptides we found no evidence for a role of c-JUN in IL-8 transcription. Instead, we show here that IL-1 and EGF within one to four hours induce expression of FRA-1 and c-FOS, which is paralled by increased recruitment of FRA-1 and c-FOS to the IL-8 promoter. Furthermore, treatment of KB cells with PD98059 completely blocked IL-1-induced c-FOS and FRA-1 expression and suppressed their recruitment to the IL-1 promoter. This results in partial inhibition of IL-1-induced IL-8 mRNA and protein expression by PD98059. In summary we provide strong evidence for a novel mechanism of IL-8 transcription that requires ERK-dependent de novo synthesis of FRA-1 and c-FOS. Recruitment of both proteins to the IL-8 promoter in addition to p65 NF-κB is required for strong and long lasting IL-8 expression.

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DEVELOPMENT AND EVALUATION OF A CUSTOMIZED DNA MICROARRAY RELEVANT TO INFLAMMATION

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Chronic inflammatory diseases are driven – at least in part - by deregulated expression of a large number of pro- or anti-inflammatory proteins. It is thus highly desirable to develop tools that allow to follow changes in the inflammatory gene expression profil. Therefore, we have designed and thoroughly evaluated a small DNA microarray that allows to analyse the expression of 135 well characterized inflammatory genes which were selected by an extensive literature search, comprising regulated proteases, matrix components, cytokines, cytokine receptors, adhesion molecules, chemokines, chemokine receptors, metabolic enzymes and signalling molecules. Three amino-modified oligonucleotides per gene were designed by an elaborated computer-based search (MWG Biotech). The specificity of the probes for their target genes was verified in more than 250 experiments using labelled cRNA prepared from 5 µg of total RNA of different cell lines, primary blood cells and tissues that had been exposed to LPS, IL-1 or TNF. Several examples of experiments will be given to demonstrate that this array allows to monitor qualitatively as well as quantitatively the inflammatory gene expression profil in mice and man. Utilizing oligonucleotides as probes, the array can be easily expanded with probes for additional genes to optimize its use in a variety of inflammatory conditions. Furthermore, the data obtained so far will be stored in a database that will be accessible by all future users of the array. In summary, our data provide an example how to rapidly develop a microarray relevant to a defined biological context at low cost and with high versatility.

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POST-TRANSCRIPTIONAL REGULATION OF IFN-γ EXPRESSION BY p38 MAPK IN HUMAN NK CELLS

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Human Natural Killer (NK) cells are a subpopulation of lymphocytes defined by expression of CD56 and lack of CD3. They play an important role in the innate immune response, for example mediating cytolytic killing of virus-infected cells or tumours. They also secrete immunomodulatory cytokines, which influence the adaptive immune system and other arms of the innate immune response. For example NK-derived interferon γ (IFN-γ) primes myeloid cells for increased production of pro-inflammatory cytokines, and promotes differentiation of T helper cells towards the Th1 phenotype. In experimental models of infectious disease, survival is often dependent upon the early synthesis of IFN-γ by NK cells. This is driven by cytokines such as IL-12 and IL-18, which are produced by pathogen activated monocytes or dendritic cells. Here we show that IL-12 and IL-18-dependent IFN-γ production by NK cells involves the stabilisation of IFN-γ mRNA by the mitogen activated protein kinase p38 pathway. The 3' untranslated region of IFN-γ mRNA mediates p38-dependent mRNA stabilisation, and contains five copies of the motif AUUUA, which has been implicated in the regulation of mRNA decay. Hence IFN-γ gene expression is post-transcriptionally regulated in a similar manner to other pro-inflammatory genes such as TNFα, IL-6, IL-8 and cyclooxygenase-2.

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REGULATION OF TRISTETRAPROLIN EXPRESSION BY THE MITOGEN-ACTIVATED PROTEIN KINASE P38 PATHWAY

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The mitogen-activated protein kinase (MAPK) p38 pathway plays a fundamental role in the regulation of inflammatory gene expression. MAPK p38 is notably implicated in the regulation of expression of Tumour Necrosis Factor α (TNFα), a major pro-inflammatory cytokine. Deregulation in TNFα synthesis is linked to many inflammatory diseases including Rheumatoid Arthritis and Inflammatory Bowel Disease. Therefore control of TNFα synthesis is a major target for therapies. In LPS-stimulated macrophages RAW 264.7 cells, p38 regulates both the on-phase and off-phase of TNFα synthesis. MAPK p38 is required for the stabilisation and translation of the TNFα message. However, MAPK p38 also regulates the expression of Tristetraprolin (TTP), a zinc finger protein which binds to and destabilises TNFα mRNA. TTP is therefore part of a negative feedback loop that functions to restrain TNFα production. We show here that p38 is required for the stabilisation of TTP mRNA; that this stabilisation is mediated by an adenosine-uridine rich element (ARE) found in the 3'untranslated region (3'UTR) of the TTP transcript; and that TTP is able to bind to its own 3'UTR therefore potentially auto-regulating its expression.

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REGULATION OF CD33-RELATED SIGLECS BY CYTOKINES

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The Siglecs are a family of immune inhibitory cell surface receptors. There are eleven members of this recently identified family of glycoproteins. Each receptor contains at least one tyrosine motif in their cytoplasmic tail. The CD33 related Siglecs (Siglecs 5-11) contain a membrane proximal ITIM motif (I/V/L/S)xYxx(L/V) and a membrane distal ITSM motif TxYxx(V/I). SH2-containing phosphatases are recruited to the receptor upon tyrosine phosphorylation of ITIMs. CD33 has been shown to bind to the phosphatases SHP-1, SHP-2 and SHIP through the two phosphorylated tyrosine motifs in its cytoplasmic tail. Although eleven Siglec family members have been identified little is known about regulation during immune response. Here we describe the regulation of Siglec expression in response to cytokines such as GM-CSF, IL-2, IL-10, IFN-γ and LPS. Real time PCR has been used to examine Siglec expression patterns in various cell lines. Flow cytometry has been used to determine expression of Siglecs on the cell surface. The data suggests that this family of inhibitory receptors are highly regulated during the immune response and thus likely contribute to the regulation of the inflammatory response.

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MUTATION & FUNCTIONAL ANALYSIS OF THE HUMAN IL-18 PROMOTER IN SARCOIDOSIS

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Sarcoidosis is a multisystemic disease of unknown aetiology, characterised by a granulomatous inflammatory process. The primary manifestation of the disorder is an accumulation of mononuclear inflammatory cells, mostly activated $CD4^+$ Th1 T-lymphocytes, which produce IL-2. IL-18 is important in the pathogenesis of sarcoidosis, via its AP-1 and NFκ-B-mediated regulation of IL-2 gene transcription and protein production. In 2/3 sarcoidosis patients spontaneous remission occurs & the remaining patients develop a progressive form of the disease. Resolution of sarcoidosis correlates with high levels of IL-18 in the lung. This study seeks to determine whether mutations in the regulatory regions (5'untranslated region & intron-1 promoter) of the IL-18 gene influence its physiological levels and thus contribute to disease phenotype. Mutation detection was performed using single strand conformation polymorphism & restriction fragment length polymorphism analyses. SNP's were identified and screened in 142 sarcoid patients and 139 healthy controls. Results indicated that the T1336 allele (intron-1 promoter region) and the CC-607 genotype (5'UTR promoter) were significantly associated with sarcoidosis. The functional consequences of these mutations were assessed using reporter gene assays, mRNA and protein expression analyses. Initial studies demonstrate a significant increase in promoter activity in response to sarcoid BAL and various stimuli.

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TREATMENT OF HELA CELLS WITH SMALL INTERFERING RNAS AGAINST HUR OR LAMIN A AND C RESULTS IN CYCLOOXYGENASE-2 EXPRESSION.

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Short interfering RNAs (siRNAs) are now used routinely to 'knockdown' specific target proteins to investigate their function in mammalian cells. We show that using an siRNA against the AU-rich element binding protein HuR, we can achieve approximately 90% reduction of HuR protein levels in HeLa cells. These HuR knockdown cells produce cyclooxygenase-2 (COX-2) mRNA and protein as well as secreting interleukin-6 and interleukin-8. Treatment of HeLa cells with siRNA against Lamin A and C also resulted in induction of COX-2 protein. Treatment of the cells with a non-specific 'Scramble' control RNA did not induce COX-2. Whilst the induction of inflammatory response genes could be relatively specific to loss of HuR or Lamin A and C, the results raise the possibility that the depletion of essential proteins from mammalian cells, or the RNA interference process itself, could cause cell stress and thereby induce the expression of inflammatory genes. This possibility needs further investigation.

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C-REL IS REQUIRED FOR CHROMATIN REMODELING ACROSS THE INTERLEUKIN-2 GENE PROMOTER

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Interleukin-2 gene transcription occurs in an activation-dependent manner in T cells responding to T cell receptor and CD28 activation. One of the critical events leading to increased IL-2 transcription is an alteration in chromatin structure across the 300bp promoter region of the gene. We initially showed that IL-2 gene transcription in $CD4^+$ primary T cells is dependent on the NF-κB family member, c-Rel but not RelA. We found that c-Rel is essential for global changes in chromatin structure across the 300bp IL-2 promoter in response to CD3/CD28 in primary $CD4^+$ T cells but not in response to the pharmacological signals, paralleling the requirement for c-Rel in IL-2 mRNA and protein accumulation. Interestingly, measurement of activation-induced localized accessibility changes using restriction enzyme digestion revealed that accessibility close to the c-Rel binding site in the CD28RR region of the promoter is specifically dependent on c-Rel. In contrast, restriction enzyme sites located at a distance from the CD28RR behave independently of c-Rel. These results suggest a non-redundant role for c-Rel in generating a correctly remodeled chromatin state across the IL-2 promoter and imply that the strength of the signal determines the requirement for c-Rel.

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NOVEL PATHWAYS FOR INTERFERON-γ-MEDIATED REGULATION OF MACROPHAGE GENE EXPRESSION

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IFN-γ regulates the expression of key genes in macrophages that are involved in the cellular uptake of lipids and the inflammatory response, two critical events in the pathogenesis of atherosclerosis. The mechanisms by which IFN-γ modulates the expression of such genes remains largely unclear. We have, therefore, investigated how IFN-γ regulates the expression of genes coding for lipoprotein lipase (LPL), which plays a key role in the pathogenesis of atherosclerosis, and the inducible cAMP early repressor (ICER), a potent transcriptional inhibitor.
IFN-γ decreased LPL gene expression at the transcriptional level and this response was mediated through three regulatory sites that bound to the transcription factors Sp1 and Sp3. Further studies revealed that IFN-γ activated casein kinase 2 (CK2), which then triggered a decrease in both the Sp1 DNA binding activity and the steady state levels of Sp3 polypeptides. In the case of IFN-γ-induced ICER expression, we showed using a panel of inhibitors against components of known signal transduction pathways that this was also mediated through CK2. The action of CK2 was via the activation of the cAMP response element binding protein (CREB), which is known to stimulate ICER gene transcription. These studies, therefore, reveal the existence of potentially novel pathways for IFN-γ regulation of macrophage gene expression that involves a CK2-mediated modulation of the action of downstream transcription factors such as CREB, Sp1 and Sp3.
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DIFFERENTIATION OF MONOCYTES TO MACROPHAGES OR DENDRITIC CELLS IS ASSOCIATED WITH CHANGES IN IRF4 AND IRF8 EXPRESSION AND DNA-BINDING CAPACITY

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IRFs form a family of transcription factors involved in cellular differentiation and innate immune responses. IRF4 expression, originally regarded as lymphocyte-restricted, has also been found in macrophages. As monocytes can differentiate into macrophages and dendritic cells (DC), we performed an RNA microarray analysis on human primary monocytes stimulated with GM-CSF and/or IL-4. IRF4 was detected as a direct target gene for both GM-CSF and IL-4. More detailed analyses of the kinetics of IRF4 mRNA expression in monocytes revealed that GM-CSF and IL-4 strongly up-regulated IRF4 mRNA expression with fast kinetics. Basal IRF4 mRNA expression was not detected in macrophages. However, IL-4, but not GM-CSF, was able to up-regulate IRF4 mRNA expression in macrophages. Instead, DC showed basal expression of IRF4 mRNA. IRF4 is known to bind DNA in a complex with IRF8 and an Ets-family member, PU.1. Based on gene targeting experiments in mice, dendritic cell differentiation and functions require IRF8/ICSBP. By DNA affinity binding experiments we found that GM-CSF, but not IL-4, enhanced DNA-binding of IRF4 and IRF8 to their target elements in monocytes. Our results indicate that during cytokine-mediated differentiation of human primary monocytes to macrophages or DCs, expression and functions of IRF4 and IRF8 are altered.

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GADD45β IS A PRIMARY RESPONSE GENE IN PANCREATIC β-CELLS EXPOSED TO IL-1β

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IL-1β is an important mediator of immune mediated apoptosis of pancreatic β-cells leading to type 1 diabetes mellitus. The apoptotic effect of IL-1β is potentiated by IFN-γ and TNF-α. JNK is crucial for IL-1β signaling of this effect as prevention of JNK activity completely blocks IL-1β induced β-cell apoptosis. In MEFs and 3DO cells induction of Growth Arrest and DNA Damage-Inducible gene (gadd)45β inhibits JNK activation. The aim of this study was to investigate gadd45β regulation by cytokines in the β-cell lines INS-1E (rat) and βTC3 (mouse).
Methods: Quantitative RT-PCR on RNA isolated from cells exposed to cytokines was performed using SYBR Green DNA-binding dye.
Results and discussion: IL-1β, IFN-γ and TNF-α induced a 10.9, 4.6 and 1.7 fold induction of gadd45β, respectively, in INS-1E cells. Time course experiments showed a significant gadd45β induction by IL-1β from 0.5h until at least 4h with a peak of 11.7 at 2h (n = 4). Preincubation of INS-1E with IL-1β and cyclohexamide caused a 3.8 fold superinduction over IL-1β treated levels. In βTC3 cells IL-1β caused a 2.9 fold induction at 1h (n = 3). In NIH-3T3 fibroblasts a 110.9 fold induction at 2h of gadd45β by IL-1β indicated insufficient gadd45β induction in β-cells. We conclude that gadd45β is a novel primary response gene in β-cell IL-1β signaling, but that induction may be insufficient to prevent proapoptotic JNK signaling.Supported by JDFRI grant #4-2002-457

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IRF-8\ICSBP CONFERS INNATE RESISTANCE TO INTERAPHAGOSOMAL PATHOGEN THROUGH THE REGULATION OF NRAMP1

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Interferon (IFN) consensus sequence binding protein (ICSBP) also known as IRF-8 is a member of a family of transcription factors termed IFN regulatory factors (IRF). Its expression is restricted mainly to cells of the immune system, and it plays a key role in the maturation of macrophages. IRF-8/ICSBP functions by forming different DNA-binding heterocomplexes with other transcription factors. Recently, using yeast two-hybrid analysis, we have isolated a novel protein that associates with IRF-8/ICSBP termed Myc-interacting zinc finger protein (Miz-1). One of the genes that is regulated by Miz-1 is Natural resistance-associated protein 1 (Nramp1), which is a proton/bivalent cation transporter. Nramp1 is located in late endosomes/lysosomes in macrophages and functions in innate resistance to interaphagosomal pathogens. A single mutation in this gene leads to mice strain sensitive to intracellular/ intraphgosomal pathogens. Using Nramp1 promoter in reporter gene assay we show that it is a target promoter of IRF-8/ICSBP following interaction with Miz-1 only in hematopoietic cell line. As a putative PU.1 binding site is present in the murine Nramp1 promoter, we tested its effect on the promoter. PU.1 is hematopoietic specific protein known to interact with IRF-8/ICSBP and has an essential role in myelopoiesis. Co-transfection of Miz-1, IRF-8/ICSBP and PU.1 resulted in an enhanced activation of Nramp1 in hematopoietic cell line. More interestingly, PU.1 enabled Nramp1 activation in non-hematopoietic cell line, therefore being the missing factor in those cells. In addition, the level of Nramp1 in macrophage cell line derived from IRF-8\ICSBP null mice is very low and can not be induced by interferon-γ and LPS. Furthermore, these mice are sensitive to a similar pathogen repertoire as mutant Nramp1 mice. These results suggest that IRF-8/ICSBP has a major role in the regulation of Nramp1 gene under pathogens infection. Our work lays the molecular basis for the restricted expression of Nramp1 and its activation by a specific subset of pathogens and suggests a possible explanation for the sensitivity of IRF-8\ICSBP$^{-/-}$ mice to interaphagosomal pathogens.

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INTERACTIONS BETWEEN NITRIC OXIDE, GLUTATHIONE AND MAP KINASES INFLUENCING CYTOKINE AND CHEMOKINE RESPONSES IN HUMAN EPITHELIAL CELLS

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Nitric oxide (NO) is an important regulator of cytokine and chemokine expression by various cell types, which may differ in susceptibility. In this study we have investigated the capacity of NO to regulate cytokine and chemokine mRNA in human A549 epithelial cells stimulated with IFN-γ, TNF and IL-1β. The mRNA species measured included TNF, IL-6, IL-8, IL-15, RANTES, IP-10 and MCP-1. Exposure of the cells for up to 24 h with the chemical NO donor SNAP did not influence the cytokine or chemokine mRNA response. Depletion of GSH with the γ-GCS inhibitor BSO rendered the cells vulnerable to NO and a consequent reduction in mRNA for TNF, IL-6, IL-15, RANTES and IP-10 was observed. However, mRNA species for MCP-1 and IL-8 remained relatively resistant suggesting differential signalling pathways between species and selectivity of NO for individual pathways. The p38 MAPK inhibitor SB203580 mimicked NO by inhibiting the induced expression of mRNA species other than MCP-1 and IL-8 whereas the IκBα kinase inhibitor BAY 11-7082 blocked all induced mRNA species. We conclude that the primary target for NO in this system is not a shared transcription factor but more probably the p38 MAPK pathway, and that high GSH levels attenuate the response.

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T CELL SPECIFIC EXPRESSION OF THE HUMAN TNF-α GENE INVOLVES A FUNCTIONAL AND HIGHLY CONSERVED CHROMATIN SIGNATURE IN INTRON 3

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Using a phylogenetic approach, we identified highly conserved sequences within intron 3 of the human tumor necrosis factor-α (TNF-α) gene. These sequences form cell type specific DNaseI Hypersensitivity (DH) sites and display cell type specific DNA-protein contacts in *in vivo* genomic footprints. Consistent with these results, intron 3 confers specific activity upon a TNF-α reporter gene in Jurkat T cells but not THP-1 monocytic cells. Thus, using a combinatorial approach of phylogenetic analysis, DH analysis, *in vivo* footprinting and transfection analysis, we demonstrate that intronic regulatory elements are involved in the cell type specific regulation of TNF-α gene expression.

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TOWARDS VIRAL-VECTOR MEDIATED ANTI-INFLAMMATORY CYTOKINE GENE TRANSFER IN EXPERIMENTAL MULTIPLE SCLEROSIS

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Multiple Sclerosis (MS) is an inflammatory disease affecting both brain and spinal cord, resulting in a relapsing, remitting pattern of clinical symptoms. The inflammatory process is mediated by several factors, including cytokines (e.g. IL-1) and reactive oxygen species (e.g. NO) that contribute to demyelination and eventually axonal damage. To counteract the inflammatory process during a chronic disease within the central nervous system, local application of viral vectors expressing an anti-inflammatory cytokine is an interesting approach.
We have produced low immunogenic adeno-associated viral vectors (AAV) and lentiviral vectors (LVV), expressing rat IL-10, rat IL-1ra or green fluorescent protein (GFP). Virally produced IL-1ra acted equipotent as recombinant IL-1ra in reducing IL-1-induced IL-6 production by C6 cells. However, virally produced IL-10 was far less potent than recombinant IL-10 in reducing lipopolysaccharide-induced TNFα production by NR8383 cells.
Administration of LVV expressing GFP into the cisterna magna (ic) of rats suffering from chronic-relapsing experimental autoimmune encephalomyelitis (cr-EAE; model for relapsing-remitting MS) results in expression of GFP in meningeal and ependymal cells of both brain and spinal cord. In a pilot study, rats suffering from cr-EAE have been injected ic with LVV expressing IL-10 (n = 3), IL-ra (n = 3) or GFP (n = 4) and promising therapeutic effects have been found.

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MECHANISMS OF STIMULUS-INDUCED CONTROL OF MRNA STABILITY

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AU rich elements (AREs) located within the 3'UTRs of many cytokines and chemokines promote rapid mRNA decay and the stability of such mRNAs is subject to modulation by both pro- and anti-inflammatory stimuli. The biochemical mechanisms underlying these processes are amenable to analysis using cell free mRNA degradation. The in vitro degradation of a capped and polyadenylated RNA substrate containing ARE sequences from the 3'UTR of the mouse KC (CXCL1) chemokine mRNA involves the sequential deadenylation of the transcript followed by 3' to 5' directed degradation of the mRNA body. The rate of in vitro decay is reduced using extracts prepared from LPS-stimulated macrophages and this response to LPS is antagonized by IL-10 or TGFβ. The deadenylation of this mRNA substrate can be selectively prolonged in intact cells by over-expression of the ARE binding protein AUF1 and extracts from such cells exhibit a comparable defect in deadenylation of the polyadenylated in vitro transcript. This process depends upon the activity of a poly A specific ribonuclease (PARN). LPS treatment of macrophages results in redistribution of PARN from the cytosol to the nucleus with resultant decrease in substrate degradation. These findings collectively suggest that alterations in specific mRNA decay are mediated through the action of ARE binding proteins and stimulus-dependent alterations in the subcellular localization of PARN activity.

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IL-4 REGULATES IFNG-INDUCED MACROPHAGE CHEMOKINE GENE EXPRESSION IN BOTH STAT6-DEPENDENT AND -INDEPENDENT FASHION

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IL-4 promotes the development of type 2 immune response in part by modulating patterns of gene expression in cells of the innate immune system. We have examined the spectrum of IL-4 mediated changes in IFNγ-stimulated gene expression using Affymetrix oligonucleotide microarray analysis of mRNAs obtained from primary thioglycollate-elicited peritoneal macrophages or RAW264.7 cells. IFNγ-treatment induced elevated expression of a broad range of mRNAs (100-200) in both cell populations and IL-4 was able to suppress the expression of approximately 10% of these changes. Thus the range of IFNγ-inducible changes that is sensitive to IL-4 is relatively restricted, consistent with our prior finding that IL-4 does not interfere with IFNγ-signaling the activation of the STAT1 transcription factor. All of the IL-4-mediated suppressive changes were lost using macrophages obtained from STAT6-deficient mice. In contrast, IL-4 promoted or potentiated the expression of a small subset of IFNγ-inducible chemokine genes including MCP-2, MCP-3, and MCP-5. This response was obtained in both STAT6+/+ as well as STAT6-/- macrophages. Interestingly, expression of the IFNγ-inducible chemokine IP-10 was suppressed by IL-4 in the presence of STAT6 and potentiated by IL-4 in its absence. These findings demonstrate that the diverse functional response to IL-4 involves signaling events that include but are not limited to the activation of STAT6 and some of which are able to cooperate with IFNγ-induced pathways.

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MODULATORY ROLE OF NFAT5 in TNF GENE TRANSCRIPTION

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The NFAT5 (TonEBP) transcription factor is a unique member of the rel/NFκB/NFAT superfamily. It activates transcription of genes involved in osmoregulation and represents the only known mammalian transcription factor that is activated in response to hypertonicity. In addition, NFAT5 was shown to be involved in the induction of the proinflammatory cytokines TNF and LT-β in response to hypertonic conditions. We show here that NFAT5 may also regulate transcription in the immune system under isotonic conditions. NFAT5 only binds to one of the six TNF NFAT binding sites that can bind the other four NFAT family members, and consistent with this, NFAT5 serves as a week activator of TNF transcription and competes for binding to its cognate site with the strong activator, NFATp, in T cells activated through TCR. Maximal levels of NFAT5 coincide with TNF and NFATp down-regulation after T cell induction by PMA + Ionophore. Furthermore, hypertonic induction of NFAT5 depresses TNF transcription in T cells after PMA + Ionophore induction. Thus, NFAT5 functions as an activator or a repressor of TNF transcription in T cells, depending on the osmolarity of the environment and the state of activation of the cells.

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PU.1 REGULATION BY GRANULOCYTE COLONY-STIMULATING FACTOR (G-CSF)

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The transcription factor PU.1 is essential for both myeloid cell and B cell development. PU.1 is induced during myelopoiesis and high levels are required for terminal maturation, yet the mechanisms regulating PU.1 gene expression are unclear. We have shown that PU.1 is stimulated in murine bone marrow cells by ex vivo G-CSF treatment. Administration of G-CSF in vivo upregulated PU.1 in the bone marrow, without causing a significant change in the proportion of Gr-1/Mac-1-positive cells. PU.1 mRNA was induced by 4-6 h, indicating transcriptional activation by G-CSF receptor signals. Stat3 plays an important role in cytokine-dependent PU.1 induction, as judged by studies using dominant inhibitory Stat3 as well as chimeric Epo receptors engineered to activate Stat3 in place of Stat5. The DNA binding activity of Stat3 was required for G-CSF-dependent PU.1 expression. Chromatin immunoprecipitation studies have shown G-CSF-dependent recruitment of Stat3 to three high affinity sites in the PU.1 promoter region. Collectively, these results demonstrate that PU.1 expression is activated by G-CSF signaling and indicate an important role for Stat3. Since high levels of PU.1 are required for myelopoiesis, these results suggest that G-CSF may stimulate a threshold level of PU.1 that is necessary for complete myeloid differentiation.

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IL-5 GENE EXPRESSION IS REGULATED BY cAMP/PROTEIN KINASE A DEPENDENT CHROMATIN MODIFICATION

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T helper 2 (Th2) cell contain relatively high levels of cyclic AMP (cAMP), a potent inducer of protein kinase A (PKA) activity.
Although treatment of T cells with compounds which elevate cAMP levels was previously described to augmente Th2 cell responses, in particular IL-5 synthesis, less is known about the adenylylcyclase/cAMP signaling pathway in Th2-lymphokine gene regulation. Using primary murine T lymphocytes we show here that (1.) inhibition of PKA activity in Th2 effector cells impairs IL-5 synthesis, whereas (2.) the expression of PKA catalytic subunit αenhances. IL-5 synthesis in Th0 cells and the thymoma cell line EL-4. In EL-4 cells the elevation of cAMP levels by forskolin induces hyperacetylation of histonr H3 at the IL-5 promoter, but not at the IL-2 promoter. Also the binding of the transcription factor NFATc1 to the IL-5 promoter depends on increased cAMP levels while the binding to the IL-2 promoter is cAMP dependent. Enhanced histone H3 acetylation at the IL-5 gene after treatment of EL-4 cells with Trichostatin A supports the binding of NFATc1 to the IL-5 promoter and elevated the levels of IL-5 secretion. Therefore, PKA activity regulates IL-5 gene expression in Th effector cells by inducing histone H3 hyperacetylation and NFATc1 recruitment.

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AUF1 SELECTIVELY REGULATES THE STABILITY OF CHEMOKINE mRNA

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Many inflammatory cytokine and chemokine mRNAs are both unstable and sensitive to stabilization in response to pro-inflammatory stimuli. AU-rich elements (AREs) within the 3'UTR of mature mRNAs confer instability and sensitivity to stabilization. These sequences are recognized by ARE-binding proteins (AUBPs) that may act to either enhance or reduce mRNA decay. Here we report that the mouse KC chemokine (CXCL1) mRNA is unstable but can be stabilized by following stimulation with cytokine IL-1 when expressed in HEK93 cells as a transgene controlled by the tetracycline promoter. Furthermore, KC mRNA can be stabilized by over-expression of the AUBP AUF1 but not HuR. This effect is sequence specific as the decay of mRNAs containing the coding region from the KC mRNA and 3'UTR sewuence of either IL-12p40 or rabbit β-globin were not stabilized. Over-expression of AUF1 decreased the rate of deadenylation leading to the accumulation of mRNAs with longer polyA tails. These observation were confirmed in a cell free mRNA degradation assay using a capped and polyadenylated *in vitro* transcript containing the ARE motif from the KC 3'UTR and extracts from 293 cells transfected to express AUF1. Extracts from HuR transfected cells were unaltered. The stabilizing effects of elevated AUF1 seen in the cell free assay were lost when the ARE motif of the substrate RNA was mutated or when a non-adenylated substrate was employed. Interestingly, binfing studies (RNA EMSA and UV-crosslinking) dmonstrated that HuR bound the KC ARE motif with greater avidity that did AUF1. These results demonstrate that the stability of ARE containing mRNAs can be selectively modulated by distinct AUBPs in a sequence specific fashion

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HIGH THROUGHPUT APPROACHES REVEAL NEW REGULATORY NETWORKS FOR IRF-8\ICSBP IN MACROPHAGE FUNCTIONING

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IRF-8\ICSBP is a member of the interferon regulatory family of transcription factors, which is essential for the differentiation of myeloid progenitor cells towards mature macrophages, and for the generation of CD8α+ dendritic cells. Mice with null mutation to IRF-8\ICSBP develop a chronic myelogenous leukemia like syndrome and are sensitive to certain pathogens. IRF-8\ICSBP exerts its activity through the interaction with other transcription factors via a specific protein-protein module termed IRF Association Domain (IAD). We have employed high throughput techniques like yeast two hybrid (Y2H) and DNA microarray in order to gain better insight on the regulatory network of IRF-8\ICSBP in mature macrophages.

Using Y2H we have identified several specific genes among which was Myc Interacting Zinc finger protein 1 (Miz-1). This interaction is restricted to immune cells and takes place on the promoter of the Natural Resistance-Associated Macrophage Protein 1 (Nramp1) gene, which functions in innate resistance to interaphagosomal pathogens. The level of Nramp1 in macrophage cell line originating from IRF-8\ICSBP null mice is very low and can not be further induced by interferon-γ and LPS. Our work lays the molecular basis for the restricted expression of Nramp1 and its activation by a specific subset of pathogens and explains in part the sensitivity of IRF-8\ICSBP$^{-/-}$ mice to interaphagosomal pathogens.

To gain better insight on the role of IRF-8\ICSBP in mature macrophages, DNA microarray was performed with RNA samples from peritoneal macrophages treated or not treated with interferon-γ and LPS. The analysis was performed with samples retrieved from IRF-8\ICSBP knockout mice and compared to wild type mice. This allowed us to identify specific roles for this factor in mature macrophage in general and inflammation in particular. Collectively, the pivotal role of IRF-8\ICSBP in innate immunity and inflammation could be demonstrated using high throughput technologies.

GENOMICS

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CHICKEN IMMUNE – RELATED GENE DISCOVERY BY ANALYSIS OF ESTS

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In order to increase the resources available in chicken, a large-scale EST project was recently undertaken. 21 different embryonic and adult tissues were used to create 64 cDNA libraries. 339,314 ESTs were subsequently sequenced and the data deposited in the chicken EST database: *http://www.chick.umist.ac.uk.* Upon assembly, 85,486 contigs were produced, 20% of which represent full-length cDNAs. BLAST homology searches found that 38% of contigs have orthologues in other species, with the remainder of the sequences representing avian – specific genes or rapidly – evolving genes. From the EST data it is estimated that the chicken genome contains around 35,000 genes.
This large EST collection provides us with a tool for discovering previously – unidentified chicken genes. We have used the available data to search for immune – related genes. Here we report the identification of several such genes, many of which were previously undescribed in EMBL. The ESTs include cytokines, chemokines, antigens, cell surface proteins, receptors and MHC-associated genes. The identification of these kinds of genes will allow further study of avian immunology and will pave the way for large-scale immune-related microarray experiments.

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ANALYSIS OF GLOBAL GENE EXPRESSION KINETICS IN ACTIVATED T CELLS

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Chronic inflammatory diseases, such as autoimmune disorders, allergies and asthma may be caused by the deregulation of genes crucial to T cell survival, signal transduction, cytokine secretion, or responsiveness to external stimuli. In the present study, human T-cell activation kinetics was studied at the transcriptome level using the Affymetrix U95A oligonucleotide arrays. The experiment involved five replicate measurements at nine time points. Qualitative expression pattern strings were defined which captured statistically significant changes in signal levels over time. A number of immunologically relevant genes showing characteristic expression patterns were selected and other genes with similar expression profiles were identified.
Previously published expression profiles for a variety of activation-responsive genes encoding transcription factors, apoptosis regulators, surface receptors or chemokines/cytokines were confirmed and refined, thus validating the statistical methods of the analysis. In addition we found previously unknown regulatory patterns for signaling molecules such as tyrosine kinases (*lck, fyn*) dual-specificity phosphatases (DSP6, DSP8) as regulators of MAPK pathway and members of distinct cytokine receptor families (IL-10Rα, IL-12Rβ). These novel findings may contribute to a better understanding of the sequence and dynamics of T-cell responses following activation and to the identification of pharmaceutically relevant targets in T cell-mediated disorders.

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IL-6 POLYMORPHISMS AND BONE DENSITY IN AUSTRALIAN MENOPAUSAL WOMEN

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Objective: To determine whether two Intereleukin-6 (IL-6) polymorphisms are associated with bone density in menopausal women.

Design: 204 Australian born menopausal women (aged 46-56 at outset) had two bone density scans of both the lumbar spine and the femoral neck over a four year period. DNA polymorphisms of the IL-6 promoter region, and the 3' region were ascertained and analysed in relation to baseline bone density, and change in bone density over time.

Results: Age, menopausal status, BMI, medications, calcium, vitamin D, cigarette and caffeine intake, and exercise levels were taken into account in the analysis (ANCOVA) of the effect of IL-6 polymorphisms on bone density. Past studies indicate that the individual polymorphism alone have a significant effect on bone density. We hypothesize that the presence of both polymorphisms will affect baseline bone density and change in bone density over time and are currently analysing the results of this study.

Conclusion: Since the regulation of IL-6 levels affects bone resorption, the determination of the involvement of IL-6 polymorphisms with bone density change will assist in the identification and treatment of individuals at risk of osteoporosis.

Key-words: Genetics; Osteoporosis; Polymorphism; Interleukin-6; Bone density, Lumbar spine, femoral neck.

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CYTOKINE INTERACTION IN DISEASE

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Abstract Text: You can either write directly into the text
Field or you can paste your text into the field. Text must not exceed 200 words.
Our study focuses on the interactions between IL-6 and IRF-1 in systemic arthritis (SA), a subgroup of Juvenile Idiopathic Arthritis (JIA). We have previously shown (in a case-control study) and confirmed (by Transmission Disequilibrium Test (TDT)) that the IL-6 -174G allele is associated with SA. We have also reported a significant association between JIA and a 3'UTR polymorphism in the IRF-1 gene. IRF-1 activates transcription of IL-6 and IL-6 induces IRF-1. We investigated the interaction between IL-6 and IRF-1 in SA.
A cohort of Caucasian SA patients and ethnically matched healthy controls were genotyped for the IL6 – 174 and IRF 3'UTR polymorphisms. The distribution of genotypes between these two loci was independent in controls but not in cases ($p = 0.006$). Logistic regression analysis done using Splus v5.1 revealed evidence for interaction between these loci ($p = 0.036$). This analysis indicates an increased risk of disease of approximately 10% to individuals with IL-6-GG, IRF-1-GA/AA in comparison with the baseline genotype of IL6-CC/CG, IRF GG.
The epistatic interaction between the previously identified high-risk genotype of IL-6 (GG) and the A-allele of IRF-1 in patients with SA provides evidence of a functional interaction between these two inflammatory genes. This interaction may also contribute to the clinical heterogeneity that characterizes SA.
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IMMUNOPHARMACOLOGY

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METHYLENEDIOXYMETHAMPHETAMINE (MDMA; "ECSTASY") INCREASES LPS-INDUCED IL-10 PRODUCTION VIA β-ADRENOCEPTOR ACTIVATION

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In the present study we examined the effect of methylenedioxymethamphetamine (MDMA; "Ecstasy") on production of the anti-inflammatory cytokine IL-10. The results demonstrate that MDMA increased LPS-induced IL-10 production. However, immunoneutralisation of IL-10 did not block the ability of MDMA to suppress IL-1β and TNF-α production, indicating that the ability of MDMA to suppress these pro-inflammatory cytokines is independent of increased IL-10 production. In vitro studies demonstrated that the MDMA-induced increase in IL-10 was not due a direct effect of the drug on immune cells. Moreover, the fact that MDMA promoted an immunosuppressive cytokine phenotype in adrenalectomised rats indicates that neither glucocorticoids nor adrenal catecholamines mediated the effect. However, pre-treatment with the β-adrenoceptor antagonist propranolol, and it's peripherally acting derivative nadolol, completely blocked the increase in IL-10, suggesting that catecholamines released in the periphery most likely from sympathetic nerves mediated the MDMA-induced increase in IL-10 production. In contrast, neither propranolol nor nadolol blocked the ability of MDMA to suppress LPS-induced TNF-α production. Thus the mechanism(s) underlying the ability of MDMA to suppress pro-inflammatory cytokine production remain to be elucidated.

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HUMAN CAFFEINE CONSUMPTION MAY INHIBIT MONOCYTE AND NEUTROPHIL CHEMOTAXIS

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The purpose of this study was to investigate the effect of caffeine and its major metabolite paraxanthine, at concentrations relevant to normal human consumption, on leukocyte chemotaxis. Following a period of abstinence (> 15hrs) from caffeine-containing food and beverages, heparinized venous blood was taken from 8 healthy volunteers (7 females). Neutrophils were isolated by density gradient centrifugation. Monocytes were isolated by density gradient centrifugation followed by enrichment using a magnetic cell separation technique. The effect of 0 – 500 μM caffeine or paraxanthine on the chemotactic response of neutrophils and monocytes to fMLP (10^{-8}M) was assessed using a Neuroprobe 48-well chemotaxis chamber. Caffeine significantly suppressed neutrophil chemotaxis (20 μM and above), and monocyte chemotaxis (50 μM and above). Paraxanthine also significantly suppressed neutrophil chemotaxis (10 μM and above) and monocyte chemotaxis (20 μM and above). In order to determine relevance to human consumption, heparinized venous blood was taken from 6 healthy female volunteers 1 hour after consumption of 2 cups of coffee and 1 bar of chocolate (approximate caffeine intake: 132mg). Using HPLC, plasma was found to contain 27 (± 2.1) μM caffeine and 10 (± 1.4) μM paraxanthine. This study demonstrates that leukocyte chemotaxis is suppressed by concentrations of caffeine and paraxanthine relevant to human consumption.

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EFFICIENT MASS PRODUCTION OF PURIFIED RECOMBINANT PORCINE IL-2 WITH BACULOVIRUS/INSECT CELL CULTURE GENE EXPRESSION SYSTEM

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We have been producing animal cytokines by baculovirus gene expression systems, known as its high efficiency and mammal like post-translational modifications. It is important to develop an efficient mass production system of purified cytokines to operate animal experiments and to establish cytokine agents. For this purpose, we attempted to use CELL MASTER (Wakenyaku Co. Ltd.), a fermentor/biorector to develop a porcine interleukin-2 (poIL-2) mass production system. The poIL-2 recombinant baculovirus infected insect cell line SF21AE was incubated in the CELL MASTER with 6 L of Sf900II serum free medium for 72 hrs. This culture fluid contained 84 mg/L (purity: 65%) of recombinant poIL-2 (rpoIL-2). Three Litres of the fluid was filtered with 300kDa ultra-filter to remove virus particles etc. and concentrated by 10kDa ultra-filtration. The rpoIL-2 in the concentrate was precipitated between 65% and 90% saturation of ammonium sulfate. The precipitate was dissolved and dialyzed against PBS. Finally, 15.5 ml of the rpoIL-2 containing fraction (5.4 mg/ml, total: 84 mg, recovery: 33%) was prepared. Purity was 94.2%.

From these results, we concluded that serum free SF21AE cell culture with the CELL MASTER and ammonium sulfate precipitation was efficient procedure for mass production of purified cytokines, especially rpoIL-2.

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CAFFEINE AND PARAXANTHINE SUPPRESS HUMAN TNF-α PRODUCTION VIA A CYCLIC AMP/PROTEIN KINASE A-DEPENDENT MECHANISM

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To date, relatively few studies have examined the effects of caffeine on the immune system. This study investigates the effect of caffeine, and its major metabolite paraxanthine, on LPS-stimulated cytokine production in human whole blood cultures. Following a period of abstinence (> 15hrs) from caffeine-containing food and beverages, heparinized venous blood was taken from healthy female volunteers. Diluted whole blood was incubated in the presence of LPS (1 μg/ml) and caffeine or paraxanthine for 24hrs. The pro-inflammatory cytokines TNF-α and IL-1β and the anti-inflammatory cytokine IL-10 were then measured in cell-free supernatants by ELISA. Whilst caffeine and paraxanthine had little or no effect on IL-10 or IL-1β production at concentrations relevant to normal human consumption (0-100 μM), significant suppression of TNF-α occurred at 50 μM and above. Also, 100μM caffeine caused a significant increase in intracellular cAMP measured by enzyme immunoassay, in adherent cells derived from whole blood. When diluted whole blood cultures were pre-treated with the specific protein kinase A (PKA) inhibitor Rp-8-Br-cAMPS (10^{-5}M and 10^{-4}M), the suppressive effect of caffeine and paraxanthine on TNF-α production was inhibited. In conclusion, this study suggests that caffeine and paraxanthine (0-100 μM) suppress LPS-stimulated production of TNF-α via activation of the cAMP/PKA pathway.

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EFFECTS OF PRE-INCUBATION WITH NITRIC OXIDE ON IL-2 PRODUCTION BY HUMAN PBMC

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Nitric oxide (NO) is a naturally occurring radical with known immune regulatory effects including inhibition of T cell proliferation. There is controversy over 1) whether NO targets early signalling events or is cytostatic/late acting and 2) whether NO inhibits cytokine production. To focus on early regulatory events we pre-stimulated human PBMC with the NO donors S-nitroso-glutathione (GSNO) or S-nitroso-N-acetyl-penicillamine (SNAP) or control non-NO releasing compounds (GSH and NAP) for up to 48 h and then studied subsequent PHA-induced proliferation (measured by 3H-thymidine uptake), release of IL-2 (by ELISA) and expression of IL-2 mRNA (by RNase protection assay). Pre-incubation with the NO donors but not control compounds time-dependently inhibited PHA-induced cell proliferation, release of IL-2 and expression of IL-2 mRNA. The inhibitory effect of NO was not accompanied by changes in CD25 expression on $CD3^+$ T cells, changes in the apoptosis marker annexin V, nor changes in expression of the housekeeping gene GAPDH. In conclusion, pre-stimulation with NO inhibits PHA-induced PBMC proliferation by a non-toxic mechanism that is associated with decreased expression of IL-2 but not CD25.

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ARTHROPOD-DERIVED HISTAMINE BINDING PROTEIN PREVENTS ALLERGIC ASTHMA

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Since histamine receptor type I blockade attenuates allergic asthma, we asked whether complete neutralisation of histamine by an arthropod-derived, high affinity histamine binding protein (EV131) would prevent allergic asthma. Intranasal administration of EV131 given prior to antigen challenge in immunised mice prevented airway hyperreactivity by 70%, abrogated peribronchial inflammation, pulmonary eosinophilia, mucus hypersecretion and IL-4, IL-5 and IL-13 secretion. The inhibitory effect of EV131 on bronchial hyperreactivity was comparable to that of glucocorticosteroids. These results demonstrate that histamine is a critical mediator of allergic asthma. Therefore, complete neutralisation of histamine, rather than specific histamine receptor blockade, has a profound effect on allergic asthma.

INFECTION

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PARTICIPATION OF THE IL-1/TL RECEPTORS IN PLAGUE PATHOGENESIS.

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Y. pestis is a causative agent of human fatal pneumonic plague. The Yop virulon enables this pathogen to survive and multiply in the lymphoid tissues. Yop delivery requires close adhesion of bacteria to target cell. Main adhesins (Inv, YadA, Ail) characteristic to *Y. pseudotuberculosis* and *Y. enterîcolitica* are not found in *Y.pestis* agent. We discovered an ability of low molecular forms (dimer-hexamer) of ^{125}I-labelled Caf1 to bind specifically to interleukin-1 receptors (IL-1R) on the target cells. Caf1 ability to interact with IL-1R testified, firstly, the participation of this antigen in the very first stages of disease development, secondly, an adhesive function of the capsule subunits, responsible for the bacterial contact with target cells, and, as a consequence: i) signal formation for initiation of expression and secretion of *Y. pestis* Yop-virulon proteins, ii) effective invasiveness. Soluble Caf1 dimers like IL-1β, induced proliferation up-regulation of human and murine thymocytes stimulated with ConA submitogenic doses. The effect of reinforced co-stimulated thymocyte proliferation under the influence of Caf1 indicated that I type receptors (IL-1RI) participated in the binding of Caf1 to IL-1R. IL-1 receptor antagonist (IL-1ra) inhibited co-stimulated human thymocyte proliferation induced by IL-1β or *Y. pestis* Caf1, thus accentuating specificity of Caf1 binding to IL-1RI. Toll-like receptor 2 (TLR2) was a member of the IL-1R family and transduced similar signals to IL-R1. Caf1 dimer like IL-1β upregulated TLR2 mRNA. Soluble Caf1 dimer was able to interact with IL-1β *in vitro*. IL-1β-Caf1 heterooligomers lost their ability to stimulate thymocyte proliferation and TLR2 mRNA synthesis.

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TIMED ABLATION OF REGULATORY CD4$^+$ T CELLS CAN PREVENT MURINE AIDS PROGRESSION

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A successful immunotherapy of murine AIDS (MAIDS) based on the elimination of replicating CD4$^+$ regulator T cells is described. We demonstrate that a single injection of the antimitotic drug Vinblastine (Vb) given 14 days post infection (pi) with LP-BM5 is able to prevent MAIDS progression. Treatment with anti-CD4 mAb at 14 days pi is similarly able to prevent MAIDS. Treatment at other time points with either Vb or anti-CD4 mAb is ineffective. The effect is based on ablation of a replicating dominantly suppressive CD4$^+$ T cell population, as indicated by adoptive transfer and *in vivo* depletion experiments using monoclonal antibodies (mAbs) against CD4 as well as combinations of mAbs against the known regulatory cell surface markers CD25, GITR and CTLA-4. Cell surface marker analysis shows a population of CD4$^+$CD25$^+$cells arising shortly before day 14 pi. Cytokine analyses show a peak in IL-10 production from day 12 to day 16 pi. MAIDS infected mice also have CD4$^+$ T cells with significantly higher expression levels of CD38 and particularly CD69, which have been demonstrated to be regulator T cell markers in the Friends retroviral model. The immunotherapy appears to prevent disease progression although no protection against re-infection with LP-BM5 is generated. This data defines a new therapy for murine retroviral infection, which has potential for use in other diseases where T regulator cell mediated immunosuppression plays a role in the disease process.

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HEPATITIS C VIRUS NON-STRUCTURAL PROTEIN 4 SUPPRESSES TH1 RESPONSES BY STIMULATING IL-10 PRODUCTION FROM MONOCYTES

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The majority of hepatitis C virus (HCV) infections become chronic, despite the presence of HCV-specific cellular and humoral immune responses. We have previously suggested that IL-10-secreting antigen-specific regulatory T cells may contribute to viral persistence, and demonstrate here that peripheral blood mononuclear cells (PBMC) from chronically HCV infected patients secrete IL-10, but not IFN-γ, in response to HCV nonstructural protein 4 (NS4). A neutralizing anti-IL-10 Ab restored this defective antigen-specific IFN-γ production *in vitro*. Furthermore, PBMC from normal individuals secreted IL-10 in response to NS4, suggesting that cells of the innate immune system, in addition to T cells, produced IL-10 in the HCV infected patients. Cell separation experiments revealed that the innate IL-10 was produced by blood monocytes, but not dendritic cells (DC). In addition, NS4 inhibited IL-12 production by PBMC in response to LPS and IFN-γ and Th1 responses to recall antigens in normal individuals. Furthermore, supernatants from NS4-stimulated monocytes inhibited LPS-induced maturation of DC and suppressed their capacity to stimulate proliferation and IFN-γ production by allo-specific T cells. Our data suggests that HCV subverts cellular immunity by inducing IL-10 and inhibiting IL-12 production by monocytes, which in turn inhibits the activation of DC that drive the differentiation of Th1 cells.

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BLOOD PLASMACYTOID DENDRITIC CELL RESPONSES TO CPG OLIGODEOXYNUCLEOTIDES ARE IMPAIRED IN HUMAN NEWBORNS

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Plasmacytoid dendritic cells (pDC) respond to unmethylated CpG motifs present in bacterial DNA or unmethylated synthetic oligodeoxynucleotides (CpG). In order to assess the function of pDC in human newborns, IFN-α production induced by CpG 2216 and phenotypic maturation of pDC in response to CpG 2006 were compared in cord blood and adult blood. We first observed that neonatal pDC displayed decreased upregulation of CD80, CD83, CD86 and CD40 whereas HLA-DR and CD54 upregulation did not differ significantly between adults and neonates. We then found that the production of IFN-α in response to CpG was dramatically impaired in cord blood. This neonatal defect was detected both at protein and mRNA levels and was still present in blood of 4 day-old babies. These findings might be relevant to the increased susceptibility of human newborns to infections as well as to the use of CpG oligodeoxynucleotides as vaccine adjuvants in the neonatal period.

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MONOCYTES ARE ACTIVATED BY *STREPTOCOCCUS PNEUMONIAE IN VITRO* IRRESPECTIVE THEIR CAPSULAR POLYSACCHARIDE STRUCTURE

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Background: *Streptococcus pneumoniae* is worldwide a major cause of invasive infections, such as pneumonia, meningitis and sepsis, which especially affect young children, elderly and the immunocompromised host. Based on antigenic differences in capsular polysaccharides, up to 90 serotypes of *S. pneumoniae* have been identified so far. We hypothesize that distinct pneumococcal capsular serotypes differently trigger an inflammatory response *in vitro*.
Objective: We investigated the capacity of distinct pneumococcal genotypes and capsular serotypes to trigger an inflammatory response *in vitro*.
Methods: Twelve clinical isolates of *S. pneumoniae* were grown to mid-logarithmic phase, harvested and heat killed at 70°C for 20 minutes. These isolates represented 6 distinct serotypes (serotypes 3, 11, 16, 19F, 23F and 33; two strains per serotype) and 9 distinct genotypes (identical genotypes within the serotypes 3, 11 and 33, respectively; distinct genotypes within the serotypes 16, 19F and 23F). THP-1 monocytes were incubated overnight with heat-killed pneumococci at a ratio of 1:100, after which IL-8 was measured in supernatants.
Results: Strains with identical capsular structure but distinct genotype strongly differed in their capacity to elicit IL-8 secretion, whereas strains with identical genotype and serotype equally activated THP-1 cells.
Conclusion: The capacity of *S. pneumoniae* to induce IL-8 release by THP-1 cells is primarily influenced by genetic differences other than the capsular gene locus.

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ENDOGENOUS G-CSF ACTIVATES NEUTROPHILS BUT BLUNTS THE PROINFLAMMATORY CYTOKINE RESPONSE IN PNEUMOCOCCAL PNEUMONIA

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Granulocyte colony-stimulating factor (G-CSF) is a cytokine that is produced during infection and considered to improve host defense via increased recruitment and performance of neutrophils and subsequent attenuation of otherwise harmful pro-inflammatory mediators. To study the role of endogenous G-CSF in the innate immune response to pneumonia, mice were treated with anti-G-CSF or control Ig and infected with *Streptococcus pneumoniae*, the most common causative pathogen in community-acquired pneumonia.
G-CSF production increased upon infection and was detectable in plasma after 48h. The absence of active G-CSF was associated with reduced activation of pulmonary PMNs (indicated by lower CD11b expression) and decreased numbers of circulating PMNs. Conversely, the production of pulmonary TNF-α, IL-1β and KC was significantly increased in anti-G-CSF treated animals after 24h. In concert, the neutralization of endogenous G-CSF did not influence PMN recruitment to the lungs, pulmonary or systemic bacterial clearance nor survival in pneumococcal pneumonia. Therefore, endogenous G-CSF exerted both pro- and anti-inflammatory properties that collectively resulted in unaltered outcome in pneumococcal pneumonia.

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LPS BINDING PROTEIN (LBP) IS A CRUCIAL COMPONENT IN THE IMMUNE RESPONSE TO *E. COLI* PERITONITIS

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LPS binding protein (LBP) is an acute phase protein that enhances the responsiveness of immune cells to LPS by virtue of its capacity to transfer LPS to CD14. To determine the role of LBP in the innate immune response to peritonitis, LBP gene deficient ($LBP^{-/-}$) and normal wild type (wt) mice were intraperitoneally infected with *Escherichia coli*, the most common causative pathogen in this disease.
LBP was detectable at low concentrations in peritoneal fluid of healthy wt mice; these local LBP levels increased rapidly upon induction of peritonitis. $LBP^{-/-}$ mice were highly susceptible to *E. coli* peritonitis, as indicated by an accelerated mortality, earlier bacterial dissemination to the blood, impaired bacterial clearance in the peritoneal cavity and more severe remote organ damage. $LBP^{-/-}$ mice displayed a diminished early TNF-α, IL-6, KC and MIP-2 production, and an attenuated recruitment of PMNs to the site of infection, indicating that acute inflammation was promoted by LBP.
Locally produced LBP is an essential component of an effective innate immune response to *E. coli* peritonitis.

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TLR2 PLAYS A ROLE IN THE EARLY INFLAMMATORY RESPONSE TO MURINE PNEUMOCOCCAL PNEUMONIA BUT DOES NOT CONTRIBUTE TO ANTIBACTERIAL DEFENSE

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Toll-like receptors (TLR) are crucial pattern recognition receptors in innate immunity. The importance of TLR2 in host defense against Gram-positive bacteria has been suggested by the fact that this receptor recognizes major Gram-positive cell-wall components, such as peptidoglycan and lipoteichoic acid. To determine the role of TLR2 in pulmonary Gram-positive infection, we first established that TLR2 is indispensable for alveolar macrophage responsiveness toward *Streptococcus pneumoniae*. Nonetheless TLR2 gene deficient mice intranasally inoculated with *S. pneumoniae* at doses varying from nonlethal (with complete clearance of the infection) to lethal displayed only a modestly reduced inflammatory response in their lungs and an unaltered antibacterial defense when compared with normal wild type mice. These data suggest that TLR2 plays a limited role in the innate immune response to pneumococcal pneumonia and that additional pattern recognition receptors likely are involved in host defense against this common respiratory pathogen.

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PROGNOSTIC SIGHNS OF EFFICIENCY OF INTERFERON PREPARATIONS IN CHRONIC VIRAL HEPATITIS C

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83 patients with chronic viral hepatitis C were under observation. Diagnosis was confirmed by detection HCV RNA in PCR. 71 patients were received interferon preparations in standart doses of usual scheme for 6 months. Control group consists of 12 patients who did not receive antiviral therapy.
Decreased ability to produce IFN-alfa and IFN-gamma, decreased immune regulator index $CD4^+/CD8^+$ with increasing of intencity of peroxide oxidation of lipids (POL) as well as of general antioxidant activity (AOA) were observed in all patients before treatment.
The first viral answer composed 36,7%, the stable one – 27,7%. Spontaneous elimination of virus in control group was not observed. The frequency of the full stable remission has depended upon both initial condition of cellular immunity and dynamic quantitive and functional indexes. Maximum efficiency therapy was registrated in patients without immunodeficit. The frequency of the full stable remission has not depended upon initial condition of interferon, system but increased to 6 month if serum IFN was decreased while ability to produce IFN-alfa and especially IFN-gamma were increased. Favourable prognostic sighns of efficiency antiviral therapy were absence initial immunodeficit, normalization IFN system with reduction of disbalance in the POL-AOA system.

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CHARACTERIZATION OF HUMAN HEMATOPOIETIC STEM/PROGENITOR CELLS INFECTED BY KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS (KSHV/HHV-8)

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Kaposi's sarcoma-associated herpesvirus (KSHV/HHV8) is a human lymphotropic herpesvirus associated with several human malignancies. The observation that these diseases predominantly involve mature hematopoietic cells led us to examine the susceptibility of human hematopoietic stem cells to HHV8 infection. Two distinct populations of stem cells, CD34 + CD38- and CD34 + AC133 +, were purified from human umbilical cord blood and adult bone marrow, exposed to semi-purified HHV8 and expanded *ex vivo* with stem cell factor (SCF), thrombopoietin (TPO) and FLT-3 ligand (FL). Both lytic and latent infections were established in the stem cells. Expression of lytic KSHV/HHV8 genes was seen in 20-30% of the cultured cells without an increase in cell death. Infected cells were characterized by flow cytometry, immuno-histochemistry. Cytokine production was measured using BioSource Multiplex Assays on the Luminex X mapplat- form. Characterization of infected stem/progenitor cells revealed that cells had maintained their stem/progenitor cell phenotype. Viral gene expression was most prevalent in cells lacking markers for mature B and endothelial cells. Secondary transmission could be achieved from the HHV8 infected stem cells. These results suggest that hematopoietic multi-potent stem cells are targets for HHV8 infection *in vivo* and play a role in the pathogenesis of HHV8 infection in part through the release of cytokines.

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REGULATION OF MONOCYTE CCR1, CCR2 AND CCR5 EXPRESSION FOLLOWING RESPIRATORY SYNCYTIAL VIRUS INFECTION

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RSV infection is the most common cause of infant hospital admission in UK. RSV pathology is characterized by chemokine secretion and pulmonary leukocyte influx. The effects of RSV on cognate chemokine receptor expression in human monocytes are unknown.
Primary monocytes, or 5-day matured Monocyte-Derived-Macrophages (MDM) were infected with RSV or exposed to conditioned media from respiratory epithelial A549 cells infected with RSV (RSV-CM). CCR1, CCR2 and CCR5 expression were assessed by RNase Protection Assay, FACS and Western Blot.
Monocytes were initially $CD14^{+ve}$, $CCR1^{-ve}$, $CCR2^{+ve}$ and $CCR5^{-ve}$. Direct RSV infection of monocytes caused a transient up-regulation of CCR1, CCR2 and CCR5 peaking at 24hrs with surface expression being lost by 48hrs. RSV-CM induced a similar pattern of CCR expression but at 96hrs post exposure, a second transient peak of CCR surface expression was detected. Data from MDMs after direct infection or RSV-CM also showed these transient peaks of CCR expression.
Specific inhibitor studies showed that the CCR cycling described requires both transcription dependant protein synthesis and a functional cytoskeleton.
These data demonstrate that RSV infection not only up-regulates chemokines but also their cognate receptors and suggests that increased CCR expression may contribute to inflammation during RSV infection.

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A METHOD FOR MAPPING CD8 EPITOPES FROM NAÏVE HUMAN DONORS

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A method has been devised for identifying CD8 epitopes from human donors. We have mapped HPV16E7 and HPV18E7 using volunteer donors (exposure to the virus is unknown). Synthetic 9-mer peptides are tested across a protein of interest. Peptides are incubated along with dendritic cells and autologous CD8 T cells. Anti-CD40 is added to boost naïve CD8 responses, and serves to increase the stimulation of DC since CD4 T cells are not present in culture. CTL Proliferation is measured using tritium incorporation. In HPV18E7, CTL activity is shown via interferon gamma ELISPOT in correlation with proliferative responses. Epitopes revealed show correlation to published data testing patients with known infections, as well as predictive binding assays. This method allows for the screening of CD8 T cell epitopes without the need for testing exposed donors. It is also more reliable source of CD8 epitopes than predictive binding assays, as CTL activity can easily be confirmed. This method of determining CTL epitopes is useful for the development of both therapeutic and preventative vaccines.

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POLYMORPHISM IN *TNFA* AND *IL4* PROMOTER REGION GENE IN RUSSIAN PATIENTS WITH CHRONIC HEPATITIS C

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Infection with the hepatitis C virus (HCV) is a common health problem and leads to a chronic disease in 60-80%. TNF-α and its increased level play a central role in pathogenesis of hepatic inflammation. Whereas, increased levels of anti-inflammatory cytokines, such as IL-4 and IL-10, do not lead to antiviral protection of host organism against hepatitis virus.

In this study 31 patients with chronic HCV infection and 52 healthy adults were examined. All examined persons were of Russian (Caucasian) origin. The polymorphic loci G-308A *TNFA* and T-590C *IL4* have been analyzed with the PCR-RFLP method.

Our results show significant increase of frequency G/A genotype of *TNFA* among HCV-patients (35,5%, p = 0,0015). The frequency of allele –308A is significantly higher in the patient's group (OR = 3,52; CI 1,12-11,48). We did not find any differences in the frequency of the T-590C polymorphism between the two groups.

Previous studies on *TNFA* associations in the German population (Hohler T. et al., 1998) and a race-mixed population (Rosen H. et al., 2002) did not show differences between chronic hepatitis C group and healthy adults.

Our work is a preliminary study for further investigations functional significance of cytokine-genes allelic variants on hepatitis C progression and possible effects of genotype-associated levels of TNF-α production on the IFN-therapy patients with hepatitis C.

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RECOMBINANT IL-18 PROTECTS AGAINST DISSEMINATED *CANDIDA ALBICANS* INFECTION IN MICE

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Endogenous IL-18 is necessary for a normal host defense against disseminated candidiasis. We have investigated the prophylactic and therapeutic potential of recombinant IL-18 (rIL-18) in a model of murine disseminated candidiasis. Prophylactic treatment of mice with rIL-18 (1 μg/mouse) one day before intravenous *C. albicans* infection (1×10^5 CFU/mouse) improved survival of the mice from 0 to 25% ($p < 0.05$). This effect was accompanied by a 10-fold decrease of fungal load in the kidneys of the rIL-18-treated mice one day after infection ($p<0.05$). In a separate experiment, administration of rIL-18 on days 5 and 7 of infection also improved the survival from 20 to 50%, in parallel with a 10 to 25-fold decrease in the fungal load ($p<0.01$). Treatment of mice with rIL-18 was associated with a two-fold increase of circulating IFNγ concentrations on day one of infection (3.9 ± 2.9 vs. 1.6 ± 1.4 pg/ml, $p < 0.05$). This resulted in an improved candidacidal capacity of both macrophages and neutrophils, which is the likely explanation for the reduced fungal load in the organs. In conclusion, rIL-18 improves the outcome of disseminated candidiasis in mice, and therefore further studies to assess its potential as adjuvant immunotherapy in fungal infections are warranted.

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INACTIVATION OF HUMAN BETA DEFENSIN 2 AND 3 BY ELASTOLYTIC CATHEPSINS

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Human β-defensin 1, 2 and 3 (HBD-1, -2 and -3) are produced by epithelial cells lining the respiratory tract and are active towards Gram positive (HBD-3) and Gram negative (HBD-1, -2 and -3) bacteria. It has been postulated that the antimicrobial activity of defensins is compromised by changes in airway surface liquid (ASL) composition in the cystic fibrosis (CF) lung, therefore contributing to the bacterial colonization of the lung by *Pseudomonas* and other bacteria in CF. In this study, we demonstrate that HBD-2 and HBD-3 are susceptible to degradation and inactivation by the cysteine proteases cathepsins B, L and S. In addition, we show that all three cathepsins are present and active in CF bronchoalveolar lavage (CF BAL). Incubation of HBD-2 and -3 with CF BAL leads to their degradation, which can be completely (HBD-2) or partially (HBD-3) inhibited by a cathepsin inhibitor. These results suggest that β-defensins are susceptible to degradation and inactivation by host proteases, which may be important in the regulation of β-defensin activity. In chronic lung diseases associated with infection, overexpression of cathepsins may lead to increased degradation of HBD-2 and -3 thereby favouring bacterial infection and colonization

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PATIENTS WITH CHRONIC MUCOCUTANEOUS CANDIDIASIS HAVE A *CANDIDA*-SPECIFIC TH2-BIASED CYTOKINE PRODUCTION

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The pathophysiologic mechanisms leading to persistent mucocutaneous infections with *Candida* species in patients with chronic mucocutaneous candidiasis (CMC) are unknown. When whole-blood from seven CMC patients was stimulated with 1×10^7 *C. albicans*, the production of IFNγ was significantly impaired compared with healthy volunteers (232 ± 120 vs. 2279 ± 609 pg/ml, $p<0.02$). In contrast, IL-10 release tended to be higher in CMC patients (677 ± 502 vs. 154 ± 32 pg/ml, $p = 0.08$). No differences in the production of IFNγ and IL-10 have been observed when blood was stimulated with either PHA or LPS (not shown). In addition, the production of TNF, IL-1β and IL-1Ra did not differ significantly between CMC patients and control subjects when blood was stimulated either with *C. albicans* (131%, 82% and 93% of normal cytokine production, $p>0.05$), LPS or PHA (not shown). As IFNγ is crucial for an efficient activation of candidacidal killing mechanisms of neutrophils and macrophages, whereas IL-10 have inhibitory effects on the function of these cells, the present data suggest that a *Candida*-specific bias of cellular immunity towards Th2 responses is important for the pathogenesis of CMC.

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ROLE OF Caf1M CHAPERON IN THE FORMATION OF CAF1 DIMERS AS *Y.PESTIS* ADHESINS INTERACTING WITH IL-1R ON CELLS

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Yersinia pestis capsular antigen Caf1 structure was studied by Fourier-transform infrared spectroscopy, differential scanning calorimetry, small angle X-ray scattering, intrinsic fluorescence, ANS fluorescence and circular dichroism. The data obtained demonstrated that Caf1 subunit was a β-structural protein which in polymer form had a high stability to thermodenaturation, acidic treatment and denaturation under reaction with guanidine chloride and urea. The dimer of Caf1 subunits was a minimal cooperative block in *Y.pestis* polymer capsule.

The Caf1 was secreted in *Y.pestis* periplasm in an unfolded state. The molecular chaperon Caf1M bound to the unfolded chain of subunit Caf1, helping in the folding, dimer formation and preventing non-productive aggregation in periplasm. Moreover, Caf1 protein present in periplasm as a complex with periplasmatic molecular chaperon (Caf1M), was represented by a dimer and tetramer. Besides, one molecule of Caf1M was present in the composition of 60 kDa complex, where Caf1 protein was represented by a dimer. In the case, when Caf1 protein was represented by a tetramer in the composition of 120 kDa complex, two Caf1M molecules were present. While examining the preparations of the periplasmatic complex (Caf1M-Caf1) using SDS-PAG–electrophoresis, Caf1M and Caf1 proteins were represented as monomers. The electrophoresis of Caf1 protein under non-denaturing conditions had shown many oligomers with even numbers of subunits, from dimers (31 kDa) to tetramers (62 kDa), hexamers (93 kDa), *etc.* These data supported the fact, that Caf1 protein polymerization was limited by a tetramer in periplasm under the control of Caf1M only in the condition of high concentration of Caf1M-Caf1 complex in solution. Caf1M then delivered the Caf1 dimer to an outer membrane molecular usher protein Caf1A responsible for translocation of the Caf1 dimers through the outer membrane. ^{125}I-Caf1 dimer effectively boud to IL-1 reseptors on immunocompetent cells.

Thus, the data obtained supported the fact that active non-pilus adhesin *Y. pestis* (Caf1 dimer) was formed via the Caf1M chaperon already being in *Y. pestis* periplasm.

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STREPTOCOCCI AND LACTOBACILLI DIFFERENTIALLY INDUCE MATURATION AND CYTOKINE AND CHEMOKINE PRODUCTION IN HUMAN MONOCYTE-DERIVED DENDRITIC CELLS

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Dendritic cells (DCs) are the most efficient antigen presenting cells and thus have a major role in regulating host immune responses. In the present study we have analyzed the ability of Gram-positive pathogenic *Streptococcus pyogenes* and nonpathogenic *Lactobacillus rhamnosus* to induce the maturation of human monocyte-derived DCs. *S. pyogenes* stimulation resulted in high expression of DC co-stimulatory molecules CD80, CD83 and CD86, while *L. rhamnosus* only moderately enhanced CD83 and CD86 expression levels. Stimulation of DCs with *S. pyogenes* also induced the production of pro-inflammatory and Th1 type cytokines and chemokines. Most importantly IL-2 and IL-12 production was seen both at mRNA and at protein level. In contrast, *L. rhamnosus* was unable to induce any notable cytokine or chemokine production in DCs. Bacteria-induced DC maturation, especially cytokine and chemokine production, was reduced when bacteria were heat inactivated. We also report that bacteria-induced DC maturation is partially dependent on bacteria-induced production of TNF-α and interferon (IFN)-α/β. Our results show that DCs have the ability to respond differentially depending on the type bacterial stimulus. While pathogenic *S. pyogenes* preferentially induced a Th1 type response, stimulation with nonpathogenic *L. rhamnosus* resulted in the development of semi-mature DCs.

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LEPTIN MEDIATES PROTECTIVE IMMUNITY AGAINST *MYCOBACTERIUM TUBERCULOSIS* INFECTION

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Leptin, product of the *ob*-gene, is a pleiotropic protein mainly produced by adipocytes. Leptin is primarily involved in basal metabolism and in addition promotes Th1 responses. In *Mycobacterium (M.) tuberculosis* infection, an adequate Th1 response is essential. To investigate the role of leptin in *M. tuberculosis* infection, we intranasally infected wild-type (WT) and leptin deficient (*ob/ob*) mice with $6x10^4$ CFU of *M. tuberculosis*. *Ob/ob mice* displayed enhanced bacterial outgrowth in the lungs after 5 and 10 weeks of infection and decreased survival ($p = 0.1$). The lungs of *ob/ob* mice showed a diffuse granulocytic inflammatory infiltrate and lung lymphocyte numbers were reduced compared to WT mice. Flow cytometric analysis of lung CD4 and CD8 positive T-cells showed that these cells were less activated in *ob/ob* mice than in WT during infection. Levels of the protective key cytokine interferon-γ were reduced in the lungs of *ob/ob* mice after 2 and 5 weeks. Functionally, *ob/ob* splenocytes were also impaired: Interferon-γ production by splenocytes stimulated with PPD was reduced. In conclusion, we show that leptin contributes to a protective Th1 immune response against *M. tuberculosis* infection.

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IMPORTANT ROLE OF INTERLEUKIN-1 SIGNALLING IN ATYPICAL MYCOBACTERIAL INFECTION

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Interleukin (IL)-1 is a potent pro-inflammatory cytokine that has been implicated in the pathogenesis of several inflammatory diseases. *Mycobacterium (M.) kansasii* is one of the most frequent non-tuberculous mycobacterial pathogens, causing pulmonary disease similar to tuberculosis in immunocompromised patients. Little is known about the pathogenesis of *M. kansasii* infection. Therefore, we intranasally infected IL-1 signalling deficient IL-1 Receptor 1 knockout (IL-$1R_1$KO) mice and C57Bl/6 (WT) mice with 3×10^4 CFU *M. kansasii*. IL-1β levels in lung homogenates were upregulated at 4 and 8 weeks post-infection. At both time-points, a strongly enhanced bacterial outgrowth was found in lungs of IL-$1R_1$KO compared to WT mice ($P < 0.005$). At the later time-point, 7 out of 8 IL-$1R_1$KO mice had disseminated disease; in the WT group 2 out of 8 mice were liver culture positive. Eight weeks post-infection IL-$1R_1$KO mice displayed higher levels of IL-1β and TNF in lung homogenates indicating more inflammation in the lungs. No differences in IL-6 or MIP-2 were found. Interestingly, levels of the anti-inflammatory cytokine IL-10 were elevated in the lungs of IL-$1R_1$KO mice at both time-points. These data suggest that the IL-1 signaling pathway and therefore IL-1 is important in host defense against murine atypical mycobacterial infection.

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THE RISE AND FALL OF INFLAMMATORY CYTOKINES IN PRE-SYMPTOMATIC INDIVIDUALS

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A study comprising of 56 human volunteers was conducted to provide data on cytokine kinetics in healthy individuals. Unexpectedly, this study has also yielded information on cytokine profiles prior to the onset of disease symptoms. Four blood samples were taken from each volunteer throughout the course of a day to provide data on diurnal variation. mRNA was extracted using Roche magnetic separation technology and cytokine profiles were analysed using real time RT-PCR. Following the study, nine volunteers succumbed to infection within 9 days of their samples being taken (URTI n = 8 and Gastro-enteritis n = 1). Two other individuals, one with hay fever and the other with elevated creatine kinase levels were also observed. These 11 volunteers repeated the study once recovered, allowing for valid data comparison between their natural levels and those seen before the onset of symptoms. Here we present consistent rises in the expression of MCP-1, IL-6 and TNF-α along with reduced expression of Fas-L, IL-8, IL-1β and IL-10, providing a fresh insight into cytokine dynamics prior to the onset of symptoms.

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RAPID GENERATION OF FUNCTIONAL HUMAN ANTIBODIES DERIVED FROM FAB/PHAGE DISPLAY LIBRARIES

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We introduce a procedure for fast generation of fully human antibodies derived from "Fab on phage" display-libraries. The technology is based on the compatibility of display-vectors and IgG expression constructs, and allows reformating of individual Fab clones to IgG, as well as reformatting of antibody repertoires. Examples of *batch reformatting* of an uncharacterized Fab-repertoire and of a pool of Fabs, previously analyzed on phage-level, are presented. The average transient expression levels of the IgG constructs in Hek293T cells are above 10 µg/ml, and allow the use of conditioned media in functional assays, without preceding antibody purification. Forthermore, we describe a high-throughput purification method yielding in IgG-amounts sufficient for initial antibody characterization. Our technology allows the generation and production of target/antigen specific complete human antibodies in a similar or even faster timeframe than raising monoclonal antibodies by conventional hybridoma techniques. The technique can be applied to a wide range of research and disease areas, for identification of new therapeutics, diagnostics and research tools.

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FAILURE TO SUPPRESS AN ANTI-INFLAMMATORY RESPONSE IN HEPATITIS C INFECTED LIVER MAY CONTRIBUTE TO VIRAL PERSISTENCE

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Immune responses to HCV infection are likely to be influenced by several factors, including local cytokine production. We have shown that a significant IFN-γ response predominates in HCV infected liver. Other studies have suggested that a Th2 response predominates, predisposing towards viral tolerance. It has also been suggested that T regulatory cells producing large amounts of IL-10 & TGF-β suppress local inflammatory responses. We therefore examined levels of the Th2 cytokine IL-10 and the T regulatory cytokines TGF-β & IL-13 in HCV infected liver. HCV-infected (n = 11) and control cirrhotic tissue (n = 20) was obtained at time of transplantation for end-stage liver disease. Normal controls consisted of tissue from donor organs (n = 12). Tissue was snap-frozen, powdered, protein was extracted and IL-10, TGF-β & IL-13 levels were quantified using a modified ELISA. Relatively high levels of both TGF-β & IL-13 were detected in normal liver tissue compared to IL-10 (25.03, 54.87 and 1.73 ng/100 mg protein respectively). This may contribute to the tolorogenic state that characterizes healthy liver. IL-10 levels are increased in HCV infected compared to normal liver (4.71 vs 1.73 ng/100 mg protein, $p < 0.005$). This increase in IL-10 may represent a physiological feedback in response to the large amounts of IFN-γ produced. TGF-β levels were significantly reduced in HCV infected and cirrhotic control tissue when compared to normal ($P < 0.05$), suggesting that TGF-β producing T regulatory cells are not involved in the suppression of Th1 responses in cirrhotic HCV liver. IL-13 levels were significantly reduced in cirrhotic controls ($P < 0.05$), but not in HCV infected compared to normal tissue, suggesting suppression of the Th2 response in cirrhotic controls. However, suppression of this Th2 response is not evident in HCV infection. In HCV infected liver, the lack of suppression of the Th2 response seen in other inflammatory conditions of the liver may represent a new component of HCV survival strategy.

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TYK-2 DEFICIENT MICE SHOW INCREASED SUSCEPTIBILITY TO *LISTERIA MONOCYTOGENES*

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Tyk2 was the first member of the Jak-family kinases to be isolated and shown to be involved in IFNAR signalling [1]. Tyk2-deficient mice show increased susceptibility to various viral infections and increased resistance against LPS induced endotoxic shock [2, 3]. In the present study we investigated the role of Tyk2 in the immune defence against *Listeria monocytogenes*.

Upon i.p injection of age and sex-matched littermates with 10^6 *L. monocytogenes* $Tyk2^{-/-}$ mice showed decreased survival rates with an unchanged bacterial burden in liver and spleen. However, histomorphological changes were drastically increased in $Tyk2^{-/-}$ mice. This increased susceptibility of Tyk2-deficient mice was associated with elevated levels of IL-6 and IFN-γ. Preliminary results of *in vitro* infections of IFN-β treated macrophages showed decreased cellular survival that was accompanied by a failure to produce NO in the absence of Tyk2.

These results indicate an essential role of Tyk2 in host defence against *L. monocytogenes* probably via dysregulation of IL-6, IFN-γ and NO production required for the activation of antibacterial mechanisms and for the preservation from pathohistological changes.

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INFLAMMATION

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BYSTANDER ACTIVATED LYMPHOCYTES : A PHENOTYPIC COMPARISON WITH RHEUMATOID SYNOVIAL LYMPHOCYTES

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Previous studies have supported a role for bystander-activated lymphocytes in cognate-dependent TNF-α production by monocytes in the joints of rheumatoid arthritis (RA) patients. Currently we are examining the phenotypic changes induced on lymphocytes during chronic cytokine exposure to determine how such cells resemble those found in the RA joint. We are particularly focussing upon chemokine receptor and adhesion molecule expression, as well as the phenotype and activation status of the expanded populations.

Lymphocytes cultured with IL-2/IL-6/TNF-α or IL-15 over 8 days demonstrated a 3-fold expansion of the NK cell population, whilst the proportion of T cells remained static. Interestingly, both populations demonstrated a 2-5-fold increase in expression of the inhibitory heterodimer NK receptor CD94/NKG2a. Other such inhibitory/activatory receptors are now being studied on these populations.

Preliminary analyses of chemokine receptor expression has demonstrated cells proliferating in response to cytokines as consistently $CCR5^+$ and $CCR6^+$, but incurring a progressive loss of CCR7 expression with lengthening exposure. These receptors bind lymphocyte chemotractants including macrophage inflammatory proteins and RANTES. This profile is consistent with the development/outgrowth of an effector memory population.

Expression patterns of these markers on synovial membrane/fluid lymphocytes from RA patients are well established. This work will more closely define pivotal cell types for cognate-dependent cytokine production in diseased joints & advance understanding of prolonged cytokine exposure in chronic inflammation.

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Upregulation of the cytokine MIF during atherogenesis and MIF-mediated destabilization of atherosclerotic plaques

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Macrophage migration inhibitory factor (MIF) is a cytokine that plays a critical role in inflammatory conditions such as septic shock, colitis, rheumatoid arthritis, or lung inflammation. Atherosclerosis (AS) is a chronic inflammatory response of the arterial wall and MIF was thus considered important in atherogenesis. MIF was found to be upregulated during the progression of human AS and was detected locally in the arterial wall. In vivo complex formation between MIF and its intracellular binding protein JAB1/CSN5 specifically occured in plaques. MIF expression in endothelial cells and macrophages was upregulated by oxLDL. Studying the role of MIF on neointima lesion formation following wire-induced injury of carotid arteries in apolipoprotein E-deficient (apoE – / –) mice revealed that MIF was upregulated in smooth muscle cells (SMC) 24h after endothelial denudation. After three weeks, MIF was predominantly found in endothelial cells and foam cells. Neutralizing MIF with a monoclonal antibody resulted in a marked reduction of neointimal macrophages and inhibited transformation of macrophages into foam cells. Conversely, the neointimal SMC content was expanded, suggesting that MIF was involved in plaque destabilization. In vitro flow assays with aortic endothelial cells revealed that plaque destabilization by MIF could be based upon MIF-mediated monocyte recruitment.

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INCREASED LEVELS OF INTERLEUKIN-10 IN SALIVA OF SJÖGREN'S SYNDROME PATIENTS. CORRELATION WITH DISEASE ACTIVITY

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Objective: To determine the levels of interleukin (IL)-10, IL-2, IL-4, interferon (IFN)-γ in saliva of patients with secondary Sjögren's Syndrome (SS) and to correlate them with laboratory and clinical parameters of disease activity.

Methods: The levels of IL-2, IL-4, IL-10, and IFN-γ were measured in salivary samples, directly obtained from Stenone duct, in 14 SS patients and 26 healthy controls by ELISA assay according to the manufacturer's instructions.

Results: A significant elevation of IL-10 level was found in salivary fluids of SS patients compared to healthy controls ($p = 0.007$). Elevated IFN-γ salivary levels were found in some SS. IL-2 and IL-4 were undetectable in all saliva samples. In SS patients IL-10 levels significantly correlated with the degree of xeroftalmia and xerostomia ($p = 0.02$ and $p = 0.01$, respectively) and with ESR ($p = 0.006$).

Conclusions: Our data suggest that elevated IL-10 levels are detectable in saliva of SS patients and correlate with the severity of the disease.

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FUNCTIONAL ANALYSIS OF COELIAC DISEASE-ASSOCIATED PROMOTER POLYMORPHISM IN THE IKBL GENE.

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Recent studies have shown potential genetic association between the human *IKBL* gene (a putative regulator of NFκB encoded in the MHC) and various autoimmune diseases. A promoter polymorphism in the *IKBL* gene was investigated due to its high frequency in CD patients. Luciferase reporter constructs were designed to compare the normal and variant DNA sequences and were transfected into the colonic epithelial cell line, HCT-116. Cells were then incubated in the presence of either (1) IL-1β, (2) TNF-α, or (3) dexamethasone for 24 hours and luciferase activity measured. When transfected into HCT-116 cells, the polymorphism altered gene expression, showing decreased activity of the mutant sequence relative to the common allele. IKBL expression was significantly decreased following stimulation of cells with IL-1β ($P < 0.001$) and TNF-α ($P < 0.01$). Conversely, gene expression was significantly increased by glucocorticoids ($P < 0.001$). Therefore, IKBL expression is downregulated by pro-inflammatory and upregulated by anti-inflammatory agents. We have previously demonstrated an inhibitory effect of IKBL on NFκB. These results indicate that IKBL expression is analogous to other known inhibitors of NFκB and supports the view that it may form part of a novel regulatory loop controlling NFκB, which may be relevant in the pathogenesis of autoimmune diseases.

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REGULATION OF IL-6 SIGNALLING IN PERITONEAL INFLAMMATION

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The mesothelium dictates the host response to local pro-inflammatory insult by orchestrating chemokine production. Using a model of SES induced peritonitis we previously identified heightened neutrophil infiltration with delayed clearance in IL-$6^{-/-}$ mice. To elucidate the mechanism directing this pro-inflammatory shift, the expression of SOCS-3 and activation of STAT1/3 was investigated in human peritoneal mesothelial cells (HPMC), murine peritoneal macrophages (Raw 264) and *in vivo* using murine peritoneal tissue.

Using immunoblot and mobility shift approaches in HPMC, we found that IL-6/ sIL-6R induced nuclear translocation of

P-STAT-3, maximal at 30 minutes, and a peak in SOCS-3 expression at 15 minutes. Stimulation of Raw264 with IL-6 or IFN-γ induced nuclear translocation of both STAT-1 and

P-STAT-3. Peak expression of SOCS-1 and SOCS-3 again preceded that of STAT-1 and 3.

In vivo, nuclear STAT-3 peaked at 3 hours (C57/Bl6 mice), in contrast to IL-$6^{-/-}$mice which peaked at 6 hours. Exposure to soluble gp130 was shown to rapidly inhibit IL-6 downstream signaling events.

The balance of STAT1/3 and SOCS activation plays a critical role in controlling the peritoneum's response to bacterial challenge and thus provides a potential target for therapeutic intervention aimed at enhancing host defence and resolving inflammation.

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PRO-INFLAMMATORY MACROPHAGES: PARTIAL DIFFERENTIATION OR PREFERENTIAL PATHWAY?

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Macrophages are a major source of cytokines in chronic inflammatory diseases such as RA. An inflammatory cytokine imbalance suggests either selective activation or selection of specific pro-inflammatory macrophages. Activation signals determine monocyte cytokine profiles but have little influence on macrophages: indeed, differentiation is more influential, the differentiation factors used would argue against selection of pro-inflammatory monocytes. M-CSF, in inflamed synovial joints, drives differentiation of infiltrating monocytes resulting in a pro-inflammatory intermediate, which upon further differentiation results in an anti-inflammatory profile. Progression to an anti-inflammatory macrophage may be blocked by other cytokines or by an increased sensitivity to apoptosis. Results show that although M-CSF macrophages are insensitive to Fas apoptosis, they are sensitive to apoptosis induced by TNFα and aged monocytes. In addition, maturation was blocked when cultured in the presence of RA-supernatants resulting in a pro-inflammatory profile upon stimulation. Description of conventional and alternatively-activated or M1/M2 differentiated macrophages lead us to investigate whether a preferential differentiation pathway exists in a chronic inflammatory setting; M1 cells were predominantly pro-inflammatory and M2 or M-CSF cells were discriminatory for IL-10 production. In conclusion, a subtle balance between apoptosis, route of differentiation and signals encountered maintains tissue macrophages in a pro-inflammatory state.

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THE INFLUENCE OF VEGF ON FUNCTIONAL ACTIVITY OF MURINE PERITONEAL MACROPHAGES

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VEGF (vascular endothelial growth factor) is an important angiogenic mediator produced by most of the tumor cells. Besides it can also induce chemotactic response of human monocytes (Barleon et al., 1996; Clauss et al., 1996). The aim of the study was to investigate the influence of VEGF on inflammatory and angiogenic activity of murine peritoneal macrophages in vitro. Murine resident peritoneal macrophages were incubated during 24 h in the presence of VEGF at concentration rate of 0,5-100 ng/ml and then tested spectrophotometrically for nitric oxide (NO), superoxide anion production (NBT-test), pinocytosis and 5'-nucleotidase activity. VEGF showed maximal effect at 50-100 ng/ml. It increased spontaneous and LPS-stimulated nitroxide anion production, spontaneous suporoxide anion production and did not influence on fluid-phase pinocytosis. VEGF also suppressed TPA-stimulated superoxide anion production and decreased the activity of 5'-nucleotidase. Incubation of macrophages in the presence of VEGF also enhanced VEGF mRNA expression as tested by RT-PCR. Taken together these data suggest that VEGF produced by tumour cells may not only attract monocytes to the tumour site but also possesses other immunomodulatory functions and can stimulate angiogenic activity of macrophages. Nitroxide anions were shown to increase endothelial cell proliferation (Ziche, 1998). Enhanced NO production and paracrine stimulation of VEGF synthesis in macrophages may produce additional loop for angiogenesis inside the tumor.

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INTERLEUKIN-1 IS A POTENT STIMULUS FOR CARDIAC FIBROBLAST MIGRATION

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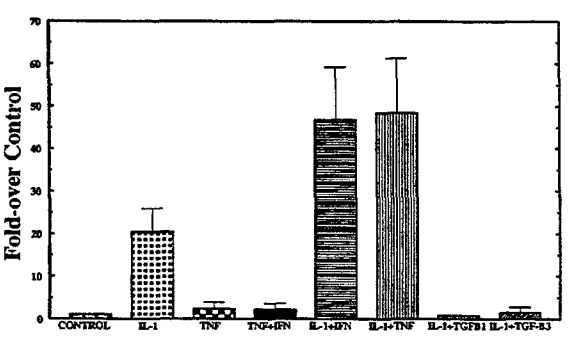
Although cytokines exert profound effects on immune cell migration, less is known about their effects on fibroblast migration. In this regard, our lab has investigated cardiac fibroblast migration after exposure to several key inflammatory cytokines and growth factors. The experimental system utilized cultures of cardiac fibroblasts isolated from neonatal rat hearts exposed to cytokine treatment for 24 hours prior to migration analysis utilizing a modified Boyden chamber. As shown in the figure, Interleukin-1 (IL-1β) is a potent stimulant for fibroblast migration, Tumor Necrosis Factor (TNFα) appears to be a weak stimulant. IL-6 and Interferon (IFNγ) produce minimal response or may even inhibit basal migration (not shown). Combination treatments have also been investigated (designed to mimic the early wound environment as well as the chronic changes known to exist in infarct and non-infarct myocardium). These investigations indicate that the IL-1β effect is augmented by the addition of IFNγ or TNFα, however it is markedly reduced by the addition of Transforming Growth Factor (TGFβ1 or β3).



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CARD15 VARIANTS ASSOCIATED WITH CROHN'S DISEASE RESULT IN IMPAIRMENT IN THE UP-REGULATION OF CHEMOTACTIC CYTOKINES

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Crohn's disease is a chronic inflammatory bowel disease, frequently associated with variants in the CARD15 gene. The protein encoded by this gene is found almost exclusively in monocytes and macrophages, and is thought to respond to bacterial muramyl dipeptide (MDP). MDP can activate NFκ-B in cells lines transfected with the normal version of CARD15, and that this response is severely abrogated in cells transfected with the variants associated with Crohn's disease. The aim of this study was to determine the normal gene expression response of human macrophages to MDP using gene expression arrays, and to elucidate any differences that occur in Crohn's disease. We demonstrate that the principle action of MDP is to up-regulate chemotactic cytokines, and illustrate that this response is deficient in patients carrying two variant copies of CARD15. We provide secondary confirmation of these results by ELISA. We subsequently demonstrate that Crohn's patients are less able to up-regulate these cytokines and recruit neutrophils to skin windows. Our findings suggest that Crohn's disease results from a defect in recruiting cells required for the acute inflammatory response. It is probable that consequent persistence of organic material in the bowel leads to granuloma formation and secondarily chronic inflammation.

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SIMULTANEOUS QUANTIFICATION OF OVER 20 CYTOKINES/CHEMOKINES IN MOUSE SERUM OR PLASMA SAMPLES USING XMAP TECHNOLOGY

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Quantification of mouse cytokines and chemokines is a challenge in many areas of biomedical research using mouse models. Conventionally, these proteins are measured individually with ELISA methods, which is costly, time-consuming and difficult for quantifying multiple analytes due to sample volume limitations. Here we report the development of a multiplexed immunoassay system for simultaneous quantification of over 20 different mouse cytokines and chemokines (including G-CSF, GM-CSF, IFNγ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-13, IL-17, IP-10, KC, MCP-1, MIP-1α, MIP-1β, RANTES, and TNFα, etc.) in a single sample. The technology includes capture of analytes by a mixture of specific antibody-immobilized microparticles, which are differentially dyed with two fluorophores. The captured analytes are detected by a cocktail of detection antibodies. Following binding of a fluorescent-labeled reporter molecule, the signal is quantified by a Luminex^{100} reader. Each antibody pair used for individual analyte is highly specific, with no or negligible cross-reactivities to other cytokines or chemokines within the panel. The standard curves range from 3.2 to 10,000 pg/mL. The sensitivities for the assays are between < 1 to 10 pg/mL in serum matrix. The assay robustness is demonstrated by excellent precision (CV (10% inter-assay; CV (5% intra-assay), linearity of dilution (100 ± 20%), and accuracy (100 ± 20%) in serum matrix. Total assay time is 2 hour for serum-free samples or overnight for serum or plasma samples. The availability of this sensitive, rapid, and robust method for simultaneous measurement of multiple analytes provides a powerful yet economic tool for both screening purpose or for accurate quantification of mouse cytokines and chemokines.

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INTERPLAY BETWEEN INTERFERON-γ (IFN-γ) AND INTERLEUKIN-6 (IL-6) SIGNALLING CO-ORDINATES LEUKOCYTE TRAFFICKING DURING INFLAMMATION

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Regulated recruitment and clearance of leukocytes is the hallmark of competent host defense, resolving inflammation. In contrast, increased residence time of activated leukocytes at sites of inflammation or infection is the hallmark of chronic inflammation. Central to this process is a shift from innate to acquired immunity characterised by the rapid recruitment of neutrophils (PMN), their apoptotic removal and replacement with mononuclear leukocytes. We now report that interplay between the IFN-γ and IL-6/sIL-6R signalling cascades facilitates PMN infiltration and subsequently promotes their apoptotic clearance. Induction of peritoneal inflammation in IFN-γ-deficient (IFN-$\gamma^{-/-}$) mice resulted in impaired neutrophil recruitment and suppressed IL-6 expression. Reconstitution of IFN-γ signalling restored PMN infiltration, IL-6 levels and CXC-chemokine expression. Modulation of PMN recruitment by IL-6 signalling was confirmed by administration of IFN-$\gamma^{-/-}$and IL-$6^{-/-}$ mice with HYPER-IL-6 (sIL-6R-IL-6 fusion protein) or IFN-γ. Although HYPER-IL-6 attenuated PMN influx in IFN-$\gamma^{-/-}$ mice, IFN-γ had no effect on PMN infiltration in IL-$6^{-/-}$ mice. Examination of the leukocyte infiltrate from IFN-$\gamma^{-/-}$ and IL-$6^{-/-}$ mice showed that apoptosis was aberrant in the absence of IFN-γ and IL-6 as a result of impaired sIL-6R-mediated signaling. These data identify a pivotal role for IFN-γ and IL-6 in regulating the shift from innate to acquired immunity through control of both the recruitment and clearance phases of PMN trafficking.

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IN VIVO COUNTER-REGULATION OF IL-1α AND IL-1ra IN MURINE KERATINOCYTES

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The potent proinflammatory cytokine IL-1α is constitutively expressed and sequestered by keratinocytes and comprises one member of the IL-1 gene family which includes IL-1β, IL-18 and the specific inhibitory molecule, IL-1 receptor antagonist (IL-1ra). In addition to IL-1α, keratinocytes also synthesise prodigious quantities of an intracellular splice variant of IL-1ra. Although homeostatic regulation of the IL-1 system in keratinocytes has long been suspected, there is currently little evidence to support this.

To explore this issue, PAM212 murine keratinocytes were exposed to increasing concentrations of either IL-1α or IL-1ra for 24 hours and ELISAs performed to assess production of the opposing ligand. IL-1ra release was induced following stimulation by mIL-1α in a dose-dependent manner (0 pg/ml to 1,924 ± 97 pg/ml with 10 ng/ml IL-1α; n = 3) and, conversely, treatment of keratinocytes with IL-1ra resulted in increased IL-1α release in a dose dependent fashion (529 ± 69 pg/ml to 1,043 pg/ml with 1 ng/ml IL-1ra; n = 3). In both cases, addition of a blocking anti-IL-1 receptor type 1 antibody inhibited release of the opposing cytokine, indicating a key role for this receptor in the feedback loop. To determine whether a similar counter-regulation occurs *in vivo*, epidermis from transgenic mice in which overexpression of IL-1α or IL-1ra was targeted to keratinocytes by the human keratin-14 (K14) promoter was analysed. In concordance with our *in vitro* findings, epidermal sheets from K14-IL-1α transgenic mice released eight times more IL-1ra than those from wildtype mice following *ex vivo* culture (5,006 ± 140 pg/ml compared with 611 ± 18 pg/ml; n = 3). Conversely, IL-1α release was raised 3-4 fold in epidermal sheets derived from two independent K14-IL-1ra transgenic lines (678 ± 35 pg/ml and 530 ± 121 pg/ml compared with 164 ± 29 pg/ml; n = 3), a response abrogated by incubation with a neutralising anti-IL-1ra antibody. Addition of specific neutralising antibodies against type I and type II IL-1 receptors to these *ex vivo* cultures indicated that the counter-regulation mechanism is mediated extracellularly through the type I IL-1 receptor alone. Finally, exposure of PAM212 keratinocytes to increasing concentrations of soluble type I IL-1 receptor (sIL-1RI) resulted in a counter-intuitive dose-dependent increase in released IL-1α protein (230 ± 33 pg/ml to 500 ± 47 pg/ml with 100 ng/ml sIL-1RI).

Taken together, these observations provide the first demonstration of counter-regulation of IL-1 receptor ligands in keratinocytes mediated via the type-1 IL-1 receptor in which production of an IL-1 agonist and antagonist are mutually interdependent. These findings underscore the importance of tight control of activity in this cytokine system in the skin.

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BYSTANDER-ACTIVATED LYMPHOCYTES IN INFLAMMATION: A NATURAL PROCESS?

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Recently we described that bystander-activated lymphocytes contribute to inflammation by inducing (in a contact -dependent manner) the production of proinflammatory cytokines from macrophages. More recently we have characterized these lymphocytes in terms of their proliferation and apoptosis kinetics, phenotype and cytokine profile.
Lymphocytes were activated with either IL-2/IL-6/TNFα or IL-15 and found to proliferate optimally after 8 days in culture following a lag phase of 2-3 days. From day 8 a decrease in proliferation was seen which corresponded with an increase in apoptosis from day 5.
Over the 8 day culture period the proportion of CD3 + ve T cells remained constant whereas the proportion of NK cells increased up to 3-fold with an increase in activation markers (CD25, CD69 and HLA-DR) on both cell types.
These bystander activated cells produced IFNγ, GM-CSF and LTα, with little IL-10 or TGFβ detected. In contrast lymphocytes activated for 48 hours with cross-linked anti-CD3 produced the anti-inflammatory cytokines IL-10 and TGFβ in addition to IFNγ, GM-CSF and LTα.
Investigation of these bystander activated lymphocytes and the relative importance of the T cells versus the NK-expanded populations will give further insight to those factors which induce and perpetuate chronic inflammatory events in disease.

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INFLUENCE OF CORTICOSTEROIDS ON CYTOKINES AND ADHESION MOLECULE LEVELS IN ACUTE PANCREATITIS

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Glucocorticoids have potent immunosuppressive and anticytokine effects. Evidence concerning the effects of glucocorticoids on leukocyte adhesion molecule expression are inconclusive.
The blood levels of IL-8, IL-17, TNF-alpha, ICAM-1, and E-selectine were studied in 28 patients with severe acute pancreatitis. The cytokines and adhesion molecule levels measured immediately after admission, at the 3rd, 7th, and 14th day. Measurement performed using ELISA technique. All patients were divided on two groups: first group compiled 16 patients, in which dexamethasone was applied in the complex management of acute pancreatitis, and control group – 12 patients that did not receive glucocorticoids. The increased levels of IL-8, IL-17, TNF-alpha, ICAM-1, and E-selectine were noted in both groups of patients. The gradually increase of all cytokines and adhesion molecule blood levels up to seventh day was noted in patients of the control group. The levels of IL-8, IL-17, TNF-alpha, ICAM-1, and E-selectine increased during first three days in patients of the first group. At the seventh day the cytokine's level stabilized and starting from the third day the gradually decrease of their levels were noted. There was a clear correlation between IL-17 and ICAM-1 plasma levels during the all period of observation.
The data presenting herein concerning the ability of dexamethasone to inhibit cytokine-induced changes of endothelial and leukocytic adhesion molecule and may also provide new insight into protective role of glucocorticoids.

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IL-18 AS AN INDICATOR OF FIBROSIS IN HCV PATIENTS

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Hepatic fibrosis is characterized by events involving cytokines. We propose that IL-18 is a prime candidate in fibrosis of HCV. In this study IL-18, fibrogenic stimulation index (FSI), procollagen type III peptide (P-III-P) and METAVIR biopsy scores were assessed in HCV patients.
Methods: IL-18 was assayed in sera obtained from patients with HCV by ELISA. Using an in vitro assay FSI was assessed in sera obtained from HCV patients (n = 20). FSI is a good index of collagen deposition in fibrosis. Serum P-III-P was assessed using RIA and liver biopsies were scored using METAVIR analysis.
Results: IL-18 levels were significantly higher ($p < 0.05$) in HCV patients with METAVIR score 3-4 compared to HCV patients with METAVIR score 1-2 and these were significantly elevated compared to controls. FSI and P-III-P levels were significantly elevated ($p < 0.05$) in HCV patients with METAVIR score 3-4 compared to HCV patients with METAVIR score 1-2.
Conclusion: Our results indicate that excellent correlations exist between IL-18 and FSI and METAVIR and between P-III-P and METAVIR. FSI can be used as an index of fibrosis and provides an assay to screen effective antifibrotic drug therapies which may be targeted at IL-18. (supported by CIHR and Health Canada).

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IL-18, FSI and P-III-P in INFLAMMATORY BOWEL DISEASE

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Inflammatory bowel disease (IBD) is a complex disease process involving cytokines and includes Crohn's disease (CD) and ulcerative colitis (UC). Collagenous colitis (CC) is characterized by inflammation and a thickened subepithelial collagen layer. In this study, IL-18 was assessed in CC, CD, UC, irritable bowel syndrome (IBS) and normal subjects and compared to the fibrogenic stimulation index (FSI) and procollagen type III peptide (P-III-P) in these patients with IBD and CC.
Methods: IL-18 was assayed in sera samples obtained from patients by ELISA. Using an in vitro assay we assessed the FSI of sera samples obtained from patients. The FSI correlates well with collagen deposition in other forms of fibrosis. In this study, sera samples were obtained from patients (n = 40) and compared to controls, and the FSI was assayed. P-III-P was assessed using RIA.
Results: IL-18 levels and FSI were significantly higher in CC patients ($p < 0.05$) compared to IBS disease controls and normal subjects.
Conclusion: Our results indicate that IL-18 and FSI are elevated in CC. FSI can be used as an index of fibrosis and in addition provides an assay to screen effective antifibrotic drug therapies which may be targeted at IL-18. (supported by NSHRF and CIHR).

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CYTOKINE PRODUCTION OF STIMULATED WHOLE BLOOD CULTURES IN RHEUMATOID ARTHRITIS PATIENTS RECEIVING ANTI-TNF AGENTS

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Rheumatoid arthritis (RA) patients have an increased susceptibility to infections, especially with intracellular pathogens. Proinflammatory cytokines are crucial components of the host defense against infections. The aim of the present study was to asses cytokine release in RA patients and whether anti-TNF therapy have an additional inhibitory effect on proinflammatory cytokine production.
Methods: In-vitro cytokine production upon bacterial stimulation of whole-blood was compared between ten RA patients receiving anti-TNF antibodies and ten healthy controls.
Results: When simulated with *Salmonella typhimurium* LPS (10 ng/ml), whole-blood from RA patients released significantly less cytokines compared with healthy volunteers: IFNγ (12.4 ± 11 vs 143.5 ± 101.2 pg/ml, $p < 0.01$), IL-6 (3907 ± 2363 vs 7040 ± 2960 pg/ml, $p < 0.01$) and IL-1β (357 ± 171 vs 1056 ± 616 pg/ml, $p < 0.01$). Similar data were observed when whole-blood was stimulated with heat-killed *Mycobacterium tuberculosis* (not shown). Anti-TNF therapy did not have an additional inhibitory effect on cytokine release after stimulation with either *S. typhimurium* or *M. tuberculosis*.
Conclusions: RA patients release significantly lower amounts of proinflammatory cytokines compared to controls. Therefore, therapeutical blockade of TNF in RA is particularly susceptible to induce a higher susceptibility to infections in RA patients.

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INFLIXIMAB AND THE CARDIOVASCULAR RISK FACTORS IN RHEUMATOID ARTHRITIS PATIENTS

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Tumor necrosis factor (TNF) increases IL-6 and CRP concentrations and induces changes in lipid metabolism. All these effects increase the cardiovascular risk of RA patients. The aim of this study is to asses how anti-TNF therapy influences these cardiovascular risk factors.
Methods: We investigated the lipid pattern from 33 patients treated with infliximab and 13 RA patients given placebo, before and after two weeks therapy. We also measured CRP and IL-6 in 24 patients from the anti-TNF group and 11 patients from the placebo group.
Results: The anti-TNF treated group showed a significant decrease in CRP (86 ± 54mg/l to 35 ± 35mg/l, $p < 0.01$), and IL-6 (88 ± 60 pg/ml to 42 ± 40 pg/ml, $p < 0.01$) concentrations. In addition, HDL-cholesterol was significantly higher after 2 weeks therapy in the anti-TNF treated group (0.86 ± 0.30mmol/l to 0.98 ± 0.33 mmol/l, $p < 0.01$), whereas triglycerides were slightly decreased (not shown). No effects was seen in the placebo group.
Conclusion: When administrated for 2 weeks to RA patients, anti-TNF antibodies (infliximab) raises the HDL-cholesterol level and lowers the CRP and IL-6 levels, thus significantly improving the cardiovascular risk profile of the RA patients.

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IL-18 AND THE THERAPEUTIC EFFECTS OF PENTOXIFYLLINE AND METABOLITE-1 IN A RAT MODEL OF INFLAMMATORY BOWEL DISEASE

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Interleukin-18 is a pro-inflammatory cytokine recently implicated in the pathogenesis of IBD. The aims of this study were to examine a) IL-18 in trinitrobenzenesulfonic acid (TNBS)-induced colitis in rats, b) therapeutic effects of pentoxifylline (PTX) and its metabolite-1 (M-1) in TNBS-colitis, and c) effects of PTX and M-1 on IL-18 in this model. Colitis was induced via intracolonic (i.c.) administration of TNBS (90 mg/kg). Treatment with PTX or M-1 commenced after 72 hours (64 mg/kg i.c. twice daily). Rats were sacrificed 0.5, 3, 7, and 14 days post-TNBS. Colonic damage, inflammation, and fibrosis were assessed by morphology damage score, myeloperoxidase activity and immunohistochemistry, respectively. IL-18 was measured in serum and colon homogenate by ELISA. Treatment with PTX or M-1 significantly reduced colon damage (PTX = 33%; M-1 = 38%; $p < 0.05$) and inflammation (PTX = 47%; M-1 = 63%; $p < 0.05$). Serum IL-18 was below detection at all timepoints (< 4pg/ml). Colonic IL-18 was elevated at 3 days compared to controls (64.1 vs. 34.3 pg/mg protein) and decreased by 7 days (43.7 pg/mg protein) post-TNBS. Results indicate that colonic IL-18 is elevated early in TNBS-colitis and that PTX and M-1 have therapeutic effects. We are currently analysing the effects of PTX and M-1 on IL-18 and fibrosis in this model. [supported by NSHRF]

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INFLAMMATORY RESPONSES TO ENDOTOXIN IN MICE AND MEN

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Endotoxin infusion has been widely used to study the inflammatory response; therefore we compared mice and men. *E. coli* type O113 endotoxin was prepared under identical conditions and used for both species. Humans were exposed to 2 ng/kg and mice to 500,000 ng/kg since these doses induced \\ 126\\1000 pg/ml of plasma IL-6 2 hours after injection. Ten normal adult male volunteers were infused intravenously, physiologic parameters measured and blood drawn at 2, 4, 6, 9, and 24 hours. Male C57\Bl6 mice (n = 4-12) were injected intraperitoneally and sacrificed at the same times. Blood was obtained via cardiac puncture. Both mice and men exhibited a similar neutrophilia which peaked between 6 and 9 hours after endotoxin exposure. Lymphopenia developed promptly with a nadir at 4 hours in both species and recovery by 24 hours in humans but not in mice. TNF and IL-6 peaked at 2 hours with return to baseline by 4-6 hours. No IL-1β was measured in humans while high levels were observed in mice. In contrast, IL-1RA was strongly induced in humans.
Mice produced about 10X more CXC chemokines than humans but both peaked at 2 hours. These data demonstrate that cytokine production in mice and men varies dramatically.

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CORRELATION OF EUCARYOTIC TRANSLATION FACTOR 5A IN ISCHEMIC HUMAN MYOCARDIAL TISSUE WITH IL-18: A MECHANISM FOR REDUCING CRONIC HEART FAILURE

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Eukaryotic initiation factor 5A (eIF-5A) facilitates translation by selectively transporting mRNAs from the nucleus to the cytoplasm. In experiments with human cell lines, we have found that one of the two human isoforms, eIF-5A1, induces apoptosis, and it may also be required for cytokine expression. In the present study, we examined the steady state mRNA expression levels of eIF-5A using real time quantitative PCR in human myocardial tissues obtained from patients undergoing coronary artery bypass grafting for ischemic disease. We also determined gene expression for IL-18, a pro-inflammatory cytokine expressed in ischemic human myocardium that plays a functional role in the loss of myocardial contractility. Expression of eIF-5A1&2 was detected in all 14 samples. Levels of eIF-5A1 mRNA varied from 7.3 to 279.4 pg/ng rRNA (mean 143.4) and were correlated with IL-18 (0.72, $p < 0.001$). In addition, eIF-5A1 proved to be 3-fold higher than eIF-5A2 when ischemia was induced *in vitro*. There was no correlation of eIF-5A with IL-18 in samples from non-ischemic, valve-replacement patients ($n = 19$). Based on these data, we hypothesize that specific inhibition of eIF-5A1 in human ischemic heart disease would reduce IL-18-mediated loss of functional myocytes and ameliorate chronic heart failure.

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REGULATION OF HAEM OXYGENASE-1 EXPRESSIONIN MONONUCLEAR CELLS BY INTERLEUKIN-10 AND LIPOPOLYSACCHERIDE

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Interleukin-10 (IL-10), is a potent anti-inflammatory cytokine, which has recently been shown to induce the expression of heam oxygenase 1 (HO-1). This stress response protein is intimated to have anti-inflammatory and cyto-protective roles within numerous cell-lines and animal models. Herein, we show that although in the RAW264.7 cell-line HO-1 expression is induced by IL-10, LPS can also stimulated the production of this enzyme. In primary human monocytes and *in vitro* differentiated macrophages, HO-1 is induced by IL-10 via STAT-3 and PI3-kinase signaling however in these cells LPS inhibits expression of HO-1. Additionally we show by the use of the HO inhibitor Zinc-(II)-Protoporphyrin-IX, IL-10 suppression of LPS induced cytokine production is independent of HO-1 activity.

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DEVELOPMENT OF SEVERE PANCREATITIS IN OBSE MICE RECEIVING IL-12 AND IL-18

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Obesity is a major risk factor for severe acute pancreatitis (AP). We developed a novel model of AP induced by injection of IL-12 and IL-18 daily for 3-5 days. Administration of this cytokine combination was lethal in 80% of obese leptin-deficient (*ob/ob*) mice, whereas no mortality was observed in their lean littermates (WT). Compared to WT mice, *ob/ob* mice receiving IL-12 and IL-18 developed significantly more severe AP, as evaluated by serum levels of amylase and by histological evaluation of the pancreas. TUNEL staining demonstrated marked apoptosis of the exocrine pancreas in *ob/ob* mice, whereas only scarce apoptotic cells were present in WT mice. Similar to observations in obese AP patients, disease in *ob/ob* mice was associated with edema and apoptosis in the small intestine, with adipose tissue necrosis and saponification, and with a dramatically increased production of IL-6 and acute-phase proteins. On the other hand, levels of IFNγ in *ob/ob* mice were reduced compared to WT mice. Nevertheless, in *ob/ob* mice, neutralization of IFNγ activity afforded complete protection from the toxic effects of IL-12 and IL-18, suggesting that obesity due to leptin deficiency might be associated with an increased susceptibility to IFNγ.

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SIMULTANEOUS QUANTIFICATION OF 14 CYTOKINES/CHEMOKINES IN RAT SERUM OR PLASMA USING xMAP TECHNOLOGY

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Rat represents an important model in many areas of biomedical research, where quantification of cytokines and chemokines will help better understanding of physiological and pathological processes. However, quantification of rat cytokines and chemokines has not been used frequently due to limited availability of rat sample volume, specific antibodies, cost and time associated with using the conventional ELISA products. Here we report the development of a multiplexed immunoassay system for simultaneous quantification of 14 different rat cytokines and chemokines (GMCSF, GRO/KC, IFNγ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 p70, IL-18, MCP-1, and TNFα) in a single sample. The methodology includes specific capture of analytes in samples by a mixture of antibody-immobilized microspheres differentially dyed with two fluorophores. The captured analytes are detected by a cocktail of biotinylated antibodies. Following binding of a fluorescent-labeled reporter molecule, the signal is quantified by a Luminex^{100} reader. Each antibody pair used for individual analyte is highly specific, with no or negligible cross-reactivities to other cytokines or chemokines within the panel. The standard curves for all analytes range from 6.4 to 20,000 pg/mL. The overall sensitivities are between < 1 t0 20 pg/mL in serum matrix. The assay robustness is demonstrated by a CV of ≤ 10 % for inter-assay precision and a CV of ≤ 5 % for intra-assay precision, by an average recovery of 100 ± 20 % for linearity of dilution, and by an accuracy of 100 ± 15 % in serum matrix. Total assay time is 2 hour for serum-free samples or overnight for serum or plasma samples. This simple, sensitive, accurate, and reproducible method for simultaneous measurement of multiple analytes is an economic and powerful tool for both target screening and accurate quantification of cytokines and chemokines in samples of rat origin.

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INCREASED LEVELS OF TGF-ß1 IN PATIENTS WITH ANKYLOSING SPONDYLITIS AFTER SPA THERAPY

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Ankylosing spondylitis (AS) is a chronic inflammatory disease of the axial joints with no satisfactory therapy. A beneficial effect has been reported after speleotherapy (exercises, hyperthermia and exposure to radon) in "Gasteiner Heilstollen" in Austria. The mechanisms underlying these effect are not clearly understood. We therefore, measured the serum levels of transforming growth factor-beta1 (TGF-ß1), a potent immunomodulator and antiinflamatory cytokine, in 83 patients with AS and 10 patients with non-inflammatory low back pain (LBP, controls) before and after therapy. The results demonstrated a significant increase in TGF-ß1 (total and active) after therapy. The mean concentration of total TGF-ß1 in AS was 28715 pg/ml and increased to 43136 pg/ml, $p < 0{,}0001$ while active TGF-ß1 increased from 77 to 1096 pg/ml, $p < 0.0001$. When the AS patients were divided into two groups according to pain reduction and improved mobility, group 1 (responders: $n = 46$) exhibited a 17 fold increase of active TGF-ß1 levels (96 to 1654 pg/ml) while group 2 (non-responders: $n = 37$) showed only a 7 fold increase (53 to 402 pg/ml). These results demonstrate a significant increase in circulating TGF-ß in patients with AS after combined spa therapy. Elevated TGF-ß1 may exert a beneficial effect through its anti-inflammatory function leading to reduction of joint pain and improved mobility.

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SOLUBLE GLUCOCORTICOID INDUCED TUMOR NECROSIS FACTOR RECEPTOR INDUCED CELL CYCLE ARREST IN MURINE MACROPHAGES

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Soluble GITR inhibits murine macrophage cell survival. To elucidate the mechanism underlying this process, we investigated the effect of sGITR on macrophage cell cycle. By FACS analysis, sGITR induced G1 phase arrest in a dose-dependent manner. Soluble GITR reduced the level of cyclin D2 and cyclin A and it increased expressions of p21 and p15, resulting in cdk2 and cdk4 activities. These results have suggested that sGITR arrested cell cycle at G1 phase by inhibiting cdk2 and cdk4 activities.

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IL-10 AND TGF-BETA HAVE DIFFERENT MECHANSISMS FOR INHIBITING CYTOKINE-INDUCED IFN-GAMMA PRODUCTION

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LPS-desensitisation is supposed to be an experimental model for "immunoparalytic" states in patients. IL-10 and TGF-β have been shown to mediate the process of LPS-desensitisation and can even directly induce an LPS-hyporesponsive state. LPS-hyporesponsive states can be overcome by IFN-γ, but the reversibility of these states by the IFN-γ-inducing cytokines IL-12/IL-18 depends on the inducing stimulus. Therefore, we aimed at comparing the mechanisms of LPS, IL-10 and TGF-β for the generation of LPS-hyporesponsive states. We demonstrated that IL-10 and TGF-β use different mechanisms for inhibiting cytokine-induced IFN-γ production. IL-10 inhibits IFN-γ production indirectly by suppressing the production of IFN-γ-inducing cytokines in contrast to TGF-β, which acts directly at the T cell level. These results were reflected by a differential reversibility of the LPS-hyporesponsive states induced by IL-10 or TGF-β, and suggest that the model of LPS-desensitisation does not fully reflect the dysfunction of immune cells observed in immunoparalytic patients.
Next, we addressed the molecular mechanisms underlying the inhibitory effects of TGF-β on IFN-γ production. TGF-β did not impair the activation of MAP kinases, Stat4 or ATF-2. Furthermore, the downregulation of T-bet by TGF-β occurred temporally delayed to its rapid inhibitory effect on IFN-γ production and can therefore not account for it.

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OVEREXPRESSION OF IL-1β INDUCES LIVER DAMAGE AND INTERSTITAL PNEUMONITIS BY ACTIVATING MACROPHAGES

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Interleukin-1 (IL-1) is a pleiotropic cytokine that affects mainly inflammation and immune responses. The IL-1 gene family consists of two agonistic forms, namely IL-1α and IL-1β, and one antagonistic cytokine. These two agonistic molecules are encoded by different genes and both differ from most other cytokines by lacking a signal sequence. Different from IL-1α, IL-1β is active in its secreted mature form, whereas its cytosolic precursor is inactive. Fibrosarcoma cells transfected with mature IL-1β fused to a signal sequence (ssIL-1β) were established and enhanced invasiveness manifested by ssIL-1β transfectants was observed. Here we show that overexpression of IL-1β by malignant cells induces liver damage and interstitial pneumonitis in syngeneic NFS mice bearing ssIL-1β tumors. Massive necrosis in the livers, exhibiting centrilobular and midzonal lesions accompanied by neutrophil infiltration, as well as interstitial pneumonitis were observed in mice bearing ssIL-1β tumors. In addition, in the spleens of mice bearing ssIL-1β tumors an increase in granulocytes and macrophages as well as myeloid progenitors was observed, indicating an extramedullary hemopoietic response. Administration of IL-1Ra as well as inactivators of Kupffer cell function, gadolinium chloride or dextran sulfate, significantly attenuated the liver injury in ssIL-1β tumor-bearing mice. This was also manifested by a reduction in the levels of liver aminotransferases. Inhibition in tumor development was also observed following treatment. Our results show the role of IL-1β both in tumor growth as well as in metabolic effects in the host.

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DIFFERENTIAL INFLUENCE OF PROCALCITONIN ON CYTOKINE GENE EXPRESSION IN IMMATURE AND MATURE MONOCYTES

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Procalcitonin (PCT), prohormone of calcitonin, is a highly sensitive and specific marker of bacterial sepsis. Despite the increasing clinical importance of PCT the knowledge about its biological functions, especially in sepsis is still rather limited.
The reduced proportion of $CD14^{++}$ HLA-DR^{+} monocytes along with the decreased monocyte responsiveness to bacterial endotoxins is observed in the course of sepsis. That's why the influence of PCT on various cytokine gene expression was estimated both in highly differentiated Mono Mac 6 ($CD14^{++}$ HLA-DR^{+}) and in immature SigM5 ($CD14^{+}$HLA-DR^{-}) monocyte lines. RT-PCR analysis of mRNAs for IL-1α, IL-1β, IL-6, IL-10, TNF-α, TGF-β_1, M-CSF, IL-6 Receptor, ICAM-1, TLR-4 and TLR-9 revealed significantly lower or absent responsiveness of SigM5 cells to endotoxin stimulation in contrast to high reactivity of Mono Mac 6 monocytes. PCT caused no significant responses in Mono Mac 6 cultures, but strongly stimulated IL-1α and IL-1β gene expression (5- and 7-fold respectively) in SigM5 cells.
Hence we demonstrated the differential influence of PCT on monocytes depending on their stage of maturation. The finding of IL-1 genes stimulation by PCT only in $CD14^{+}$HLA-DR^{-} monocytes might partially explain its harmful effects observed in septic but not healthy animals.

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THE AMEBIC ANTI-INFLAMMATORY MONOCYTE LOCOMOTION INHIBITORY FACTOR (MLIF) MODIFIES THE NF-κB NUCLEAR TRANSLOCATION IN HUMAN MONO

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MLIF, an anti-inflammatory peptide (Met-Gln-Cys-Asn-Ser) produced by *E.histolytica* – or its 97% pure synthetic construct – inhibits the PMA-induced expression of MIP-1alpha, MIP-1beta, I-309 chemokines and the CCR1 receptor. We studied the effect of MLIF upon NF-κB nuclear translocation. To 6×10^6 U-937 cells in RPMI, 8.6×10^{-5} M synthetic MLIF, 8×10^{-8} M PMA (induced), or neither (constitutive) or both were added and incubated from 20 min to 48 hs. Nuclear extracts were obtained and processing by EMSA method. At 24 hrs, MLIF inhibited 44% and 12% the constitutive and the induced expression of NF-κB translocation respectively ($p < 0.001$). At 48 hs MLIF inhibited 25% and 28% the constitutive and the induced expression of NF-κB nuclear translocation respectively ($p < 0.001$). On the other hand at early times (0-24 hs) MLIF *per se* was able to activate some NF-κB translocation. Super shift analysis showed the presence of p50/p50 homodimers and p65/p50 heterodimers in the NF-κB product. MLIF may thus act early over the NF-κB with an efficient inhibition at 24 hs of the constitutive levels of translocation and more vigorous late (48 hs) inhibition of induced NF-κB translocation. This pattern may be related to features of the unusual inflammatory cell trafficking observed in invasive amebiasis.

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IL-20: promoter analysis and characterization of biological function

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IL-20 belongs to the IL-10 family and has been shown to be involved in the pathogenesis of keratinocyte proliferation *in vivo*. To understand the gene regulation of human IL-20, we identified the human IL-20 promoter region. Eight fusion genes containing different regions upstream of exon 1 linked to a luciferase reporter gene were expressed in Madin-Darby canine kidney (MDCK) epithelial-like cells.. We identified a fusion gene (pE) containing 279 bp upstream of exon 1 that showed promoter activity 14-fold greater than that of the negative control. Both GM-CSF and IL-10 showed up-regulation of the promoter activity of IL-20 and induced IL-20 transcripts in monocytes. Analysis of IL-20 promoter from 198 individuals, both healthy individuals and patients with SLE, showed that there is a 318 bp insertion-deletion polymorphism on the IL-20 promoter. Insertion of this 318 bp in the promoter region decreased its promoter activity by 37% to 72%. We demonstrated that IL-20 induced production of IL-6 and TNF-α in monocytes; up-regulated the transcripts of keratinocyte growth factor (KGF), IL-6, and TNF-α in CD8-positive T cells; and induced the production of reactive oxygen species (ROS) from CD8-positive T cells.

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IL-1 ALPHA IS THE CAUSE OF IBD IN BALB/C MICE

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RAC – DEPENDANT REGULATION OF IL-10 AND VEGF PRODUCTION IN HUMAN MACROPHAGES

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The small GTPase Rac has been implicated in numerous different cellular functions such as cytoskeletal reorganisation, phagocytosis, cell migration, integrin complex formation and cell adhesion, gene transcription, cell cycle progression and proliferation. However its role in the regulation of cytokine production has not been examined in any detail. To investigate the role Rac plays in cytokine production in human macrophages we constructed an adenoviral construct expressing both wild type Rac2 and a dominant negative version (D57N) version of Rac2 which cross-dominates both Rac1 and Rac2.
We have shown, using the D57N Rac virus that the induction of pro-inflammatory cytokines TNFα and IL-6 by LPS is independent of Rac activation. However, the production of the anti-inflammatory cytokine IL-10 and the angiostatic factor VEGF were dependant on Rac activity. Furthermore using in an *in vitro* cell system model of rheumatoid arthritis, we were also able to show a Rac-dependant production of VEGF, however in this system IL-10 production was no longer dependant on Rac. The mechanisms of this Rac dependent regulation of cytokine regulation will be discussed further.
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IL-20 AND IL-22 IN PSORIASIS

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IL-20 and IL-22 are two recently identified members of the IL-10 family that includes IL-19, IL-24, and IL-26. IL-20 binds and activates IL-20RA/IL-20RB and IL-22R/IL-20RB, while IL-22 signals through the IL-22R/IL-10RB heterodimeric receptor. The transgenic (Tg) mice expressing either IL-20 or IL-22 from a variety of promoters exhibit neonatal lethality and a shiny skin appearance. Histological analysis of the skin of Tg mice shows a thickened epidermis, hyperkeratosis, and immune cell infiltration, a phenotype that resembles human psoriasis.
Both IL-20 and IL-22 activate the human keratinocyte cell line HaCaT *in vitro*, assessed using a STAT luciferase reporter assay. Subcutaneous administration of IL-22 causes epidermal thickening and immune cell infiltration, which can be neutralized by IL-22RA2, the natural soluble receptor of IL-22.
The receptors of IL-20 and IL-22 (IL-20RA, IL-20RB and IL-22R) are expressed in both lesional and nonlesional human psoriatic skin, while the ligands IL-20 and IL-22 are each up-regulated in the lesional psoriatic skin, as measured by *in situ* hybridization and quantitative RT-PCR.
Psoriasis is a T cell-mediated inflammatory skin disease. Since both IL-20 and IL-22 are expressed by activated T cells, induce a skin phenotype (thickening epidermis and immune cell infiltration), are active on keratinocytes, and are elevated in the lesional region of human psoriatic skin, they may contribute to the pathogenesis of skin diseases such as psoriasis. An antagonist of either IL-20 or IL-22 might, therefore, represent a therapeutic for such diseases.

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ANTI IL-10 THERAPEUTIC STRATEGY USING THE IMMUNOMODULATOR AS101 IN MURINE SEPSIS: DEPENDENCE ON TIMING OF IMMUNOMODULATING INTERVENTION

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The role of IL-10 in experimental sepsis is controversial. The non toxic immunomodulator AS101 has been previously shown to inhibit IL-10 expression at the transcription level. We show in this study that treatment of mice subjected to cecal ligation and puncture (CLP) with AS101 12 h after, and not before CLP, significantly increased survival of septic mice. This was associated with a significant decrease in serum IL-10 and in IL-10 secreted by peritoneal macrophages 24-48 h after CLP. At that time the ability of these cells to secrete TNF-alpha and IL-1beta, promptly suppressed in control mice, was restored in AS101 treated mice. The increased survival of AS101 treated mice was due to the inhibition of IL-10 since co-treatment with rmIL-10 abolished AS101's protective activity. AS101 increased class II antigen expression on peritoneal macrophages, severely depressed in control mice. This was accompanied by a significant elevation in the level of IFN-gamma secreted by splenocytes. Moreover, AS101 remarkably ameliorated bacterial clearance in the peritoneum and blood and decreased severe multiple organ damage. We suggest that agents like AS101 with the capacity to inhibit IL-10 and stimulate macrophage functions, may have clinical potential in the treatment of sepsis. provided they are administered during immune suppression.

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DIFFERENTIAL REGULATION OF PROSTAGLANDIN E BIOSYNTHESIS BY INTERFERON-G IN COLONIC EPITHELIAL CELLS

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Background and aims: Altered cytokine expression is part of the inflammatory response and contributes to the pathogenesis of infammatory bowel disease (IBD). Cytokine-induced cyclooxgenase (COX)-2 and the regulation of specific prostaglandin production during inflammation has not been fully investigated. COX-2 expression and activity in response to pro-inflammatory cytokines IL-1α/β, TNFα and IFNγ was evaluated in a colonic epithelial cell line, HT29.
Methods: COX-2 mRNA and protein levels were determined by Northern and Western analysis, respectively. PGE_2 production was measured by ELISA.
Results: IL-1α/β and TNFα induced concentration and time-dependent up-regulation of COX-2 mRNA, protein and prostaglandin (PG)E_2 synthesis. Co-stimulation of either TNFα or IL-1 with IFNγ resulted in reduced COX-2 mRNA and protein expression. TNFα-induced PGE_2 biosynthesis was significantly enhanced by the simultaneous addition of IFNγ and was COX-2 dependent. The combination of IFNγ and TNFα induced the microsomal prostaglandin E synthase (mPGES), coordinate with the enhanced PGE_2 synthesis.
Conclusions: These results suggest that, in terms of PGE_2 biosynthesis, IFNγ plays a *negative* regulatory role at the level of COX-2 expression and a *positive* regulatory role at the level of mPGES expression. This may have important implications for the clinical use of IFNγ in IBD.

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PLASMA CONCENTRATION OF MACROPHAGE INHIBITORY CYTOKINE-1 (MIC-1) AND THE RISK OF CARDIOVASCULAR EVENTS IN WOMEN

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Backgound: Macrophage inhibitory cytokine-1 (MIC-1) is a recently described divergent member of the TGF-β superfamily. Elevated serum MIC-1 levels occur in a number of disease states and may also be detected in all normal individuals.
Methods: We performed a prospective study among 27,628 American women. Baseline blood samples were obtained. Study endpoints were a cardiovascular event involving the cerebral or myocardial vasculature. MIC-1 serum levels were determined and compared between evetn and control groups as well as with other inflammatory mediator levels.
Findings: MIC-1 levels were higher at baseline among women who developed cardiovascular events (685 vs 578 pg/ml; $P < 0.001$). MIC-1 levels above the 90th percentile (> 856 pg/ml) indicated a significant increase in risk of an event (RR = 2.7, 95% CI 1.64-4.95, P = 0.001). The predictive value of serum MIC-1 estimation was indépendant and additive to that of CRP. Elevation of both MIC-1 and CRP indicated a relative risk of an event of 4.3 (95% CI 2.0 to 9.1, P = 0.001).
Interpretation: Baseline levels of MIC-1 were elevated independently of other markers among those who developed a cardiovascular event. This suggests that MIC-1 is marking another factor in plaque evolution that is not sampled by the other inflammatory markers. Further studies should be undertaken to determine if MIC-1 has a rôle in the clinical management of patients with vascular disease as suggested by this paper.

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DOES ANTI-INFLAMMATORY TREATMENT PREVENT THE AGE-RELATED CHANGES IN THE HIPPOCAMPUS?

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Among the reported effects of ageing is an increase in concentration of the pro-inflammatory cytokine, interleukin-1β (IL-1β). Here we investigate the role of the putative anti-inflammatory combination of dexamethasone and vitamin D_3 on age-related changes in rat hippocampus. Dexamethasone (1μg/ml) and vitamin D_3 (0.1μg/ml) was administered in drinking water to male Wistar rats, aged 3 and 22 months old (n = 6 for all groups), for 2 weeks. Hippocampal concentrations of IL-1β and IL-10 were assessed by ELISA on homogenates prepared from these treatment groups. IL-1β concentration was significantly increased in hippocampus of aged, compared with young, rats as previously shown. The present data indicate that treatment with dexamethasone and vitamin D_3 did not alter IL-1b concentration in hippocampus of young rats but it reduced the increase observed in aged rats. The concentration of the anti-inflammatory cytokine, interleukin-10 (IL-10), was significantly decreased in aged, compared with young rats and treatment with dexamethasone and vitamin D_3 resulted in a reversal of the age-related change. The data indicate that inflammatory changes observed in hippocampus of aged rats are abrogated by dexamethasone and vitamin D_3.
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IL-1 RECIPROCALLY REGULATES TIMP3 AND ADAMTS PROTEINASES IN ARTICULAR CARTILAGE

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Degradation of cartilage is a severe problem in osteoarthritis and rheumatoid arthritis. Catabolism of its major soluble polymer, the proteoglycan aggrecan, occurs in arthritis. Two related proteinases are implicated: ADAMTS (a disintegrin and metalloproteinase domain with thrombospondin motif)-4 and -5. Their activity is opposed by the tissue inhibitor of metalloproteinase (TIMP)-3. Therefore the balance between ADAMTS-4, and -5 and TIMP-3 is likely to be important for the net activity of the enzymes. The regulation of ADAMTSs and TIMP-3 was investigated in cartilage explants and chondrocytes.
ADAMTS-4 gene expression was induced by IL-1 in both human and animal (bovine or porcine) chondrocytes. ADAMTS-5 expression was constitutive in human but IL-1 induced in animal chondrocytes. In all chondrocytes studied IL-1 decreased the expression of TIMP-3 mRNA and protein. IL-1 induced the release of glycosaminoglycans (GAGs) strongly in porcine cartilage explants but only weakly in human explants. Parallel changes in ADAMTS-mediated aggrecan cleavage were demonstrated by neo-epitope assay. TGFβ increased the expression of both ADAMTS-4 and TIMP-3, but did not cause GAG release. These results suggest that aggrecan cleavage is dependent upon the expression of ADAMTS-4, ADAMTS-5 and TIMP-3. Presumably the balance between ADAMTSs and TIMP-3 is critical for cartilage degradation.

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CHARACTERIZATION OF PLAQUE-DERIVED T LYMPHOCYTES IN PATIENTS WITH CAROTID ATHEROSCLEROSIS.

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Ongoing inflammatory reactions within atherosclerotic plaques are thought to be crucial determinants of atherosclerosis. We have recently demonstrated that intracellular expression of TNF-α, IFN-γ and IL-4 in peripheral blood T lymphocytes is a marker of carotid artery disease. Our study was aimed to characterize T cell population infiltrating the atherosclerotic plaque.
Carotid plaques and peripheral blood samples were obtained from 7 patients with carotid atherosclerosis undergoing endarterectomy (3 patients had uncomplicated lesions, 4 patients had complicated lesions). Phenotypic analysis and intracellular expression of pro-inflammatory (TNF-α, IFN-γ, IL-6) and anti-inflammatory (IL-4, IL-10) cytokines were determined by flow cytometry.
Our results demonstrated that the majority of T cells were CD4+, CD45RO + and TCRα+/β+. Intracellular TNF-α and IFN-γ expression was higher in intralesional than in circulating T lymphocytes, especially in patients with complicated plaques. A positive correlation was found between TNF-α and IFN-γ expression, and among IL-4, IL-6 and IL-10 expression.
Our preliminary results confirm that the predominant T lymphocyte population in advanced human atherosclerotic lesions is CD3+ CD4+ and suggest that TNF-α and IFN-γ are crucial mediators of inflammatory intra-plaque reactions. The higher expression of these two cytokines in patients with complicated plaques confirms their involvement in the immunological mechanisms underlying plaque instability.

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TESTICULAR INTERSTITIAL AND TUBULAR CELLS PRODUCED SIMILAR LEVELS OF IL-1 FOLLOWING IN VIVO INJECTION OF LPS.

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Introduction: Endocrine and paracrine factors were shown to be involved in the regulation of spermatogenesis. IL-1 system was demonstrated in different testicular cells including, Leydig, Sertoli and germ cells. These cytokines are involved in the regulation of testicular functions and to mediate systemic pathological and inflammatory diseases.
Materials and methods: Mature mice (6-8 week-old) were intraperitonealy injected with lipopolysaccharide (LPS) (2,20,100 μg/100 μl/mouse). Three or twenty four hours later, the testes were picked, tunica albugina was removed and the testes were homogenized. In parallel some testes the seminiferous tubules and the interstitial tissue were mechanically separated. The seminiferous tubules were homogenized and the cells of the interstitial tissues were centrifuged. The precipitated cells were lysed, by three cycles of thawing and freezing, and homogenized. The levels of IL-1α, IL-1β and IL-1ra were evaluated in the homogenates and cell lysates using specific commercial ELISA kits. Total protein was determined by Biorad reagent.
Results: The levels of IL-1α and IL-1β were increased in the homogenates of testicular tissues following LPS injection, in a dose-dependent manner. IL-1β levels were higher than IL-1α. The levels of these cytokines were higher after 3 hours of LPS injection than 24 hours. However, the levels of IL-1ra remain constant following LPS injection. The levels of IL-1α and IL-1β, in the seminiferous tubules and in the interstitial tissue cells, were similar in the levels and in their response to LPS.
Conclusion: In the testis, both interstitial and seminiferous tubular cells could response to pathological conditions. It is not yet clear whether seminiferous tubules directly or indirectly affected by LPS. The response of the testicular tissue to LPS injection was by increasing both IL-α and IL-1β, but not IL-1ra. Our results may indicate that systemic infection/inflammation may affect testicular function. The testicular function could be influenced by local production of autocrine and/or paracrine inflammatory cytokines, which may affect spermatogenesis and male fertility.

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INCREASED GLOMERULAR AND EXTRACELLULAR MALONDIALDEHYDE LEVELS IN DIABETIC GLOMERULOSCLEROSIS

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Results from animal experiments have suggested that reactive oxygen species (ROS) play an important role in tissue damage associated with diabetes. To determine whether ROS are involved in patients with diabetic nephropathy, we have measured the plasma and urinary levelsof malondialdehyde (MDA), an important marker of lipid peroxidation, and assessed the immunoreactivity of MDA and superoxide dismutase (SOD) in glomeruli of patients and rats with diabetic nephropathy. The results showed that both plasma and urinary MDA levels were significantly higher in patients with diabetic glomerulosclerosis (DGS) than those of diabetic patients without proteinuria and normal controls. In DGS patients, the plasma MDA was significantly correlated with urinary MDA($p < 0.05$). The urinary MDA, but not plasma MDA, was significantly correlated with the degree of glomerulosclerosis and the index of mesangial expansion (both $p < 0.01$) in DGS patients. The immunostaining score of glomerular MDA and SOD were also significantly higher in DGS patients than in control kidneys. In rats with diabetes for more than one month, the glomerular immunostaining for both MDA and SOD were also significantly higher than in controls rats, and both were increased with the progression of diabetes. Our results suggest that oxidative stress is involved in the pathogenesis and the progression of diabetic glomerulosclerosis.

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EFFECT OF CYCLOFERON ON CYTOKINE PRODUCTION BY MONONUCLEARS OF HEALTHY VOLUNTEERS

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One of the key parameters in the immune response development is the cytokine expression profile in response to the IFN-inducing drugs. In this aspect the capacity of Cycloferon (the low molecular weight inducer of interferon) to induce the production of TNFα, IL-1 and IL-8 by blod mononuclears of the healthy volunteers was studied. The effect of Cycloferon on cytokine production, induced by standard mitogen phytohemagglutinin (PHA), was studied in parallel.
It was semonstrated that during the first 24-48 hours the PHA induces the great increase in production of TNFα, IL-8, IL-1 and INFα as well. The PHA-mediated boost in TNFα production was registered in 9 out of 10 volunteers, whereas the boost in IL-8 was registered only in 3 out of 6.
Concurrently, the capability of Cycloferon to mediate the dose-response inhibiting effect on PHA-mediated spontaneous production of IL-1β and production of TNFα and IL-8 in cells was demonstrated. The conclusion was made that Cycloferon is capable of enhancing the production of anti-inflammatory cytokine (IL-10 ot TGFand IL-8 in cells was demonstrated. The conclusion was made that Cycloferon is capable of enhancing the production of anti-inflammatory cytokine (IL-10 ot TGFβ), which inhibits the production of pro-inflamatory cytokines. The elicited effect of Cycloferon open up new vistas to its administration as anti-inflammatory drug.

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GENE EXPRESSION PROFILE OF HUMAN MONOCYTE-DERIVED DENDRITIC CELLS TREATED CONCOMITANTLY WITH LPS AND IL-10: CXCL13 INDUCED AND TRIGGERS MIGRATORY RESPONSE OF B CELLS

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Dendritic cells (DC) can express different functional programs in response to microenvironmental signals. In this study, DCs were exposed for 2 or 8 hours to Lipopolysaccharide (LPS) or Interleukin -10 (IL-10) or a combination of both stimuli. The gene expression profile of treated cells was analyzed using GeneChip oligonucleotide microarrays, representing approximately 12,600 human genes and ESTs (Affymetrix®). The two antagonistic stimuli generated two considerably different expression profiles, quantitatively and qualitatively. Furthermore, the association of LPS and IL-10 modulated a proper set of genes compated ti single treatments. IL-10 was able to antagonize 19 LPS-modulated genes after 2 hours and 29 after 8 hours on one hand, and to synergize with LPS to modulate 40 gene after 2 hours and 34 after 8 hours on the other hand. Here we show that CXCL13 mRNA, coding for a potent B cells chemo-attractant, was expressed at a higher level, and the protein was produced in higher amounts in the supernatants of LPS + IL-10 treated DCs, compared to untreated DCs or to DCs treated with either LPS or IL-10 alone. Furthermore, supernatants od DCS treated with LPS + IL-10 were able to trigger *in vitro* migration of B lymphocytes. Taken together, our data provide further informations about the effects of IL-10 on the humoral immunity.

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SECRETED APP ACTIVATES MICROGLIA VIA THE JNK PATHWAY AND STIMULATES IL-1β

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Activation of immune mechanisms is linked to several neurodegenerative disorders including Alzheimer's disease. Cleavage to the amyloid precursor protein (APP) by α- or β-secretase yields large fragments (sAPP) that elevate markers of inflammation in microglia. Here we show that sAPP utilizes a JNK-dependent signal transduction pathway for microglial activation and stimulates IL-1β production, a proinflammatory cytokine. SAPP was produced in a prokaryotic expression system and purified by two chromatography steps. Primary cultures of rat microglia were treated with sAPP; protein lysates were assayed by western blot analysis for IL-1β expression or JNK activation (using a phosphoto-specific antibody). At 30 nM, sAPP evoked activation of JNK within 30 min. Over a longer time-course, IL-1β was also stimulated in response to sAPP. A JNK inhibitor (SP600125) dose-dependently blocked activation of microglia by sAPP, as reflected in the accumulation of nitrite, the expression of inducible nitric oxide synthase (iNOS), and IL-1β production. In conclusion, our data indicate that sAPP activates the JNK pathway to effect activation of microglia, including IL-1β production, a process that could result in compromised neuronal function or survival. Supported by NIA funds (AG12411 and AG17498).

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LIPOPOLYSACCHARIDE-BINDING PROTEIN (LBP) DOES NOT PLAY AN IMPORTANT ROLE IN EARLY HOST DEFENSE DURING MURINE PULMONARY TUBERCULOSIS (TB)

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Background: Lipopolysaccharide binding protein (LBP) facilitates binding of lipoarabinomannan, a major proinflammatory cell wall component of mycobacteria, to the pattern recognition receptor CD14, resulting in activation of immunocompetent cells. The role of LBP in host defense against TB is unknown.
Aim and Methods: To determine the role of LBP in TB, LBP deficient (LBP-/-) and wild type (WT) mice were intranasally inoculated with 10^5 Colony Forming Units *M. tuberculosis*(H37Rv). Mice were monitored for survival or sacrificed after 2 and 6 w_{ee}ks for determination of mycobacterial outgrowth and host inflammatory responses. Statistics by Kaplan Meier and Mann-Whitney U test.
Results: Survival of LBP-/- and WT mice was similar (55% and 64% resp. after 29 weeks). Moreover, at 2 and 6 weeks, no difference in outgrowth of *M. tuberculosis* (lungs, liver), pulmonary leukocyte counts, or histology was seen between LBP-/- and WT mice. Also, at 2 weeks, TNF, IL-6 and IFN-γ concentrations were similar in lung homogenates of both mouse strains. At 6 weeks however, TNF and IL-6 levels were enhanced in LBP-/- mice vs. WT mice ($P < 0.05$) whereas no difference in IFN-γ was seen γ.
Conclusion: Lipopolysaccharide-Binding Protein does not play an important role in early host defense during murine pulmonary tuberculosis.

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PLASMINOGEN ACTIVATOR INHIBITOR TYPE I DEFICIENT MICE SHOW AN IMPROVED EARLY HOST DEFENSE IN *ESCHERICHIA COLI* PERITONITIS BY A NEUTROPHIL DEPENDENT MECHANISM

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Background: Abdominal sepsis is an important cause of death and plasma levels of plasminogen activator inhibitor type I (PAI-1) correlate with a poor outcome.
Aim and Methods: To determine the role of PAI-1 in abdominal sepsis, PAI-$1^{-/-}$ and normal wild type (PAI-$1^{+/+}$) mice received an i.p. injection with *E. coli* (10^4 CFU's). In some experiments, neutrophils were depleted with an anti Ly-6G mAb before peritonitis induction. Data are mean ± SE. Statistics by Mann-Witney-U, t-and log-rank tests.
Results: Peritonitis revealed elevated PAI-1 concentrations in both peritoneal fluid (PLF) and plasma of PAI-$1^{+/+}$ mice 20h postinfection ($p < 0.05$ vs 0h and 6h postinfection). PAI-$1^{-/-}$ mice showed a decreased bacterial outgrowth in PLF 6h and 20h post-infection (20h, PLF: PAI-$1^{+/+}$ $1.5 \times 10^9 \pm 3.2 \times 10^8$ vs. PAI-$1^{-/-}$: $4.1 \times 10^8 \pm 2.1 \times 10^8$ ($p < 0.05$), which was associated with an increased neutrophilic influx into the PLF 20h postinfection (PAI-$1^{+/+}$: $7.0 \times 10^5 \pm 2.1 \times 10^5$ vs. PAI-$1^{-/-}$ $2.59 \times 10^6 \pm 1.1 \times 10^6$) ($p < 0.05$). The increased neutrophil recruitment contributed to the enhanced bacterial clearance in PAI-$1^{-/-}$ mice, since neutrophil depleted PAI-$1^{-/-}$ mice did not differ from PAI-$1^{+/+}$ mice in their capacity to clear *E. coli*.
Conclusion: PAI-1 impairs host defense during *E. coli* peritonitis through inhibition of neutrophil recruitment to the site of infection.

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SIGIRR, A NEGATIVE REGULATOR OF IL-1R-, TLR4- AND TLR9-MEDIATED SIGNALING

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The Toll/IL-1 receptor superfamily plays crucial roles in the immune response by differentially recognizing pathogen products and eliciting appropriate responses. These receptors alter gene expression, mainly through the activation of NFκB and AP1. SIGIRR, a member of this family that does not activate these factors, instead negatively modulates responses. Inflammation is enhanced in SIGIRR-null mice as measured by enhanced chemokine induction after IL-1 injection and a reduced threshold for lethal endotoxin challenge. Cells from SIGIRR-null mice show enhanced activation in response to either IL-1 or certain Toll ligands. Finally, biochemical analysis indicates that SIGIRR binds to the Toll/IL-1R signaling components in a ligand-dependent manner. Our data reveals that SIGIRR functions as a biologically important modulator of Toll/IL-1R signaling.

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IL-10 PLAYS A MAJOR ROLE IN THE ENHANCED SUSCEPTIBILITY TO PNEUMOCOCCAL PNEUMONIA IN MICE RECOVERED FROM INFLUENZA A INFECTION

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Background: Influenza infection predisposes to a bacterial superinfection of the lung, which is associated with an exaggerated inflammatory response as reflected by excessive cytokine and chemokine production. Since IL-10 has been shown to hamper bacterial clearance during primary pneumococcal pneumonia and since IL-10 production (50-fold increase) is more pronounced than other soluble mediators (up to 20-fold increase) during postinfluenza pneumonia, we hypothesized that IL-10 contributes to the enhanced susceptibility to bacterial pneumonia after recovery from influenza.
Methods: C57BL/6 mice were inoculated intranasally (i.n.) with 10 TCID50 influenza A/PR/8/34. On day 14 after viral infection, mice were inoculated with 5000 cfu *S.pneumoniae* (serotype 3) i.n. One hour prior to bacterial infection, mice were treated with a neutralizing antibody against IL-10 (1mg/mouse, i.p.) or isotype control. Mice were sacrificed 48 hours after bacterial infection or used in a survival experiment. Bacterial load was determined by culturing on blood-agar plates, viral load was determined by real-time PCR; cytokines by ELISA. Data are expressed as median values. Statistics by Mann-Whitney U or Kaplan-Meier.
Results: At day 14, influenza A was cleared from the lungs, and mice had recovered clinically. Mice recovered from influenza infection demonstrated an enhanced pneumococcal outgrowth when compared to mice not previously exposed to influenza. Bacterial outgrowth was strongly reduced in anti-IL-10-treated mice compared to control mice ($3.4 \times 10^{\circ}7$ cfu vs $2.1 \times 10^{\circ}8$ cfu resp, $p = 0.02$). Pulmonary levels of TNF-α, IFN-γ and IP-10 were lower in anti-IL-10-treated mice than in control mice (all $p < 0.05$), but not those of IL-6 and KC. Importantly, mice treated with anti-IL-10 were relatively protected against secondary bacterial pneumonia as reflected by enhanced survival ($p = 0.0005$).
Conclusion: IL-10 plays a major role in the enhanced susceptibility to secondary bacterial pneumonia after recovery from influenza as reflected by enhanced survival and reduced bacterial outgrowth in mice passively immunized against this cytokine.

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OPPOSING ROLES OF p50 AND c-REL IN THE REGULATION OF CYTOKINE PRODUCTION AND PROLIFERATION IN NK CELLS.

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The Nuclear Factor-κB (NF-κB) transcription family has been well studied in regard to innate and adaptive immunity. Various family members have a role in the regulation of cell proliferation, cytokine secretion, trafficking and formation of memory. However, the role of NF-κB specifically in NK cells is less clear, with most studies focusing on cytotoxicity. Studies using mice transgenic for Inhibitor of κBα (IκBα(ΔN)) show that when multiple members of this transcription family are inhibited, NK cells are deficient in secretion of IFN-γ and are unable to proliferate in response to cytokine stimulation in vitro. The use of NF-κB -/- mouse strains, further show how two family members, p50 and c-Rel oppose each other functionally to differentially regulate IFN-γ production and cell proliferation in these innate cells. In the absence of c-Rel, NK cells have a defect in their ability to secrete IFN-γ, as well as a reduced capacity to proliferate. In contrast, the absence of p50 allows NK cells to produce higher levels of IFN-γ and proliferate significantly better than NK cells from wild-type controls. Together these data reveal individual roles for each NF-κB family member in the function of NK cells.

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CAMPYLOBACTER INDUCED CYTOKINE RESPONSES FROM AVIAN CELLS

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Campylobacter jejuni is a major cause of human inflammatory enteritis. During the course of human disease numerous pro-inflammatory cytokines are produced. Little is known, however, about the cytokine responses produced during interaction of this bacteria with the avian host. *Campylobacter* is thought of as a commensal of the avian host and the differences in innate responses between human and avian hosts may lead to a greater understanding of the disease process. We have identified a range of cytokines which are produced in response to *Campylobacter* infection of avian primary and continuous cell lines. The data would indicate that *Campylobacter* are able to stimulate the avian host. These results have implications for how we view the interaction of *C. jejuni* with its human and avian hosts.

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CYTOKINE AND CONTACT-DEPENDENT ACTIVATION OF NK CELLS BY VIRUS-INFECTED MACROPHAGES

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NK cells participate in innate immune responses by secreting IFN-γ and by destroying virus-infected cells. Here we have studied interaction between influenza A or Sendai virus-infected macrophages and NK cells. We observed a rapid, cell-cell contact-dependent production of IFN-γ from NK cells cultured with virus-infected macrophages. Expression of MICB, a ligand for NK cell activating receptor NKG2D, was up-regulated in virus-infected macrophages suggesting a role for MICB in the activation of *IFN-γ* gene expression in NK cells. IL-12Rβ2, IL-18R, MyD88 and T-bet mRNA synthesis was enhanced in NK cells cultured with virus-infected macrophages. Up-regulation of these genes was dependent on macrophage-derived IFN-α. In contrast to IL-12Rβ2, expression of WSX-1/TCCR, a receptor for IL-27, was reduced in NK cells cultured with virus-infected macrophages. IL-27R down-regulation was dependent on macrophage-derived IFN-α. Furthermore, macrophage-derived cytokines induced changes in NK cell chemokine receptor profile. *CCR1* gene expression was enhanced whereas *CXCR3* gene expression was reduced in NK cells in response to IFN-α. In conclusion, our results show that virus-infected macrophages activate NK cells through cytokines and direct cellular interactions and further emphasize the role of IFN-α in the activation of innate immunity.

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THE INNATE IMMUNITY PROTEIN LACTOFERRIN IS CLEAVED AND INACTIVATED BY CATHEPSINS

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The innate immunity protein lactoferrin is a normal constituent of airway secretions with important antimicrobial properties. Lactoferrin inhibits microbial growth by sequestering iron essential for microbial respiration and may decrease oxidant-mediated tissue injury by binding iron necessary for hydroxyl radical formation. It has recently been shown that lactoferrin can inhibit *P.aeruginosa* biofilm formation. Cathepsins are lysosomal proteases released by activated macrophages. We have previously shown that cathepsins B, L and S cause proteolytic cleavage and inactivation of another innate immunity protein, the anti-protease secretory leucoprotease inhibitor (SLPI). Incubation of lactoferrin (both iron-saturated and iron- unsaturated forms) with cathepsins B, L and S resulted in proteolytic cleavage of both proteins as demonstrated by SDS-PAGE and Western blotting. We have demonstrated the presence of active cathepsin B, L and S in CF BAL and supplementation of CF BAL with lactoferrin resulted in it's proteolytic cleavage. Using radial diffusion assays, we showed that iron-unsaturated lactoferrin loses its bactericidal activity against *P.aeruginosa* strain PAO1 when cleaved by cathepsins. We have also demonstrated that cathepsin-cleaved lactoferrin cannot inhibit LPS-induced IL-8 expression. These findings indicate the possible involvement of cathepsins in the diminution of the lung antimicrobial screen which may potentiate lung infections.

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THE ACUTE PHASE RESPONSE IMPAIRS HOST DEFENSE AGAINST *ACINETOBACTER BAUMANNII* PNEUMONIA IN MICE

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Background *Acinetobacter baumannii* is an emerging pathogen in hospital-acquired pneumonia. Trauma and post-surgical patients, displaying profound acute phase protein responses (APR), are susceptible for acquiring pneumonia. In mice, turpentine injection induces a sterile inflammation resulting in an APR.
Methods To study the influence of the APR on pulmonary host defense, mice were injected subcutaneously with either turpentine or saline in both hind limbs two days before intranasal inoculation with *Acinetobacter*.
Results Turpentine-injected mice demonstrated weight loss and an APR ($P < 05$ vs saline). Pulmonary clearance of *Acinetobacter* was impaired in the turpentine-injected mice ($P < 05$). Lung pro-inflammatory cytokine and chemokine levels (TNFα, IL-6, KC) were markedly reduced after turpentine injection (all $P < 001$), while IL-10 levels were similar. Neutrophil influx and myeloperoxidase activity in the lungs were significantly lower after turpentine injection ($P < 05$), indicating an impaired neutrophil migratory response. Moreover, mice with an APR had lower levels of total protein in their bronchoalveolar lavage fluid ($P < 05$) and showed less inflammation upon histological examination.
Conclusion The APR significantly impairs the local inflammatory response to *A. baumannii* pneumonia, which may facilitate bacterial outgrowth.

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IN VIVO TLR9 STIMULATION REDUCES THE COLITIS-INDUCING POTENTIAL OF CD4$^+$CD62L$^+$ CELLS IN THE SCID TRANSFER MODEL OF COLITIS

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There is compelling evidence that bacterial constituents influence induction and perpetuation of intestinal inflammation. We have previously shown that pretreatment of mice with CpG-motifs of bacterial DNA was protective in different models of IBD. To identify mechanisms controlling this prophylactic effect we used the SCID-transfer model of colitis. When CD4$^+$CD62L$^+$cells were transferred from CpG-oligodeoxynucleotides (CpG-ODN) treated donor-mice to SCID-hosts they only developed mild colitis in contrast to controls receiving cells from GpG-ODN or untreated donors. (8 weeks after transfer: Weight change in % / Histologic score (0-4): untreated donors: -8.2 ± 5.0/ 3.1 ± 0.3 (control), CpG-ODN treated donors: $+ 14.3 \pm 5.0/1.2 \pm 0.7$). IFN-γ secretion from mesenterial lymph node cells (MLC) of mice transferred with cells from CpG-ODN treated donors was 1000-fold lower compared to mice transferred with cells from untreated donors. Further, Rag1-deficient mice which were transferred with cells from TLR9-deficient mice developed more severe intestinal inflammation than mice transferred with cells from wt controls. (Histologic score 6 weeks after transfer: TLR9-/-: 2.9 ± 0.6; wt: 1.3 ± 0.6). Moreover, IL-6 and IFN-γ secretion from host MLC was significantly enhanced (15-fold and 4-fold). Thus, physiologic or therapeutic TLR9-ligation contributes to the formation of a T-cell population with a decreased potential to induce Th1-mediated intestinal pathology.

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CYTOKINE EXPRESSION DIFFERENCES IN CATTLE BREEDS THAT DIFFER IN HOST RESISTANCE TO THE PROTOZOAN PARASITE *THEILERIA ANNULATA*

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Theileria annulata, a tick-borne protozoan parasite, causes the disease tropical theileriosis in cattle and is a major constraint on the improvement of cattle farming in endemic areas e.g. North Africa and India. Imported, high yield European cattle e.g. Holsteins, are extremely susceptible to the parasite and infection results in high levels of mortality. In contrast, some indigenous breeds, e.g. Sahiwals, are relatively resistant to the disease.
Macrophages are the principal host cell for *T. annulata* during the acute stage of the disease. Previous work has suggested that the control of cytokine responses by macrophages is key to the observed difference in pathogenesis. Quantitative and semi-quantitative RT-PCR analyses of *T. annulata* infected macrophage cell lines derived from experimentally infected animals have revealed phenotypic differences between Sahiwals and Holsteins. However, the expression of the principal candidate cytokines, IL-1β, IL-6 and TNF-α do not exhibit breed specific differences. In contrast several genes including IL-10, iNOS and TGF-β2 were expressed at higher levels in Holstein derived lines. To further investigate the mechanism(s) involved in the macrophage regulation of disease pathogenesis we aim to generate a bovine macrophage microarray at the ARK-Genomics unit (www.ark-genomics.org) at the Roslin Institute.

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ENHANCED ERADICATION OF NONCAPSULATED *K. PNEUMONIAE*, BEARING MANNOSE-CONTAINING O-ANTIGEN FROM MOUSE LUNG DUE TO RECOGNITION BY PHAGOCYTES AND SURFACTANT PROTEIN D

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Klebsiella spp cause community-acquired bacterial pneumonia in immunocompromised individuals. Important virulence factors of *K. pneumoniae* are the capsular polysaccharide (CPS) of capsulated strains and O-antigen in the lipopolysaccharide (LPS) of noncapsulated phase variants. Lung innate immunity components such as the sugar binding proteins surfactant protein D (SP-D) and macrophage mannose receptor (MR), play an important role in rapid recognition and elimination of the pathogen from the upper respiratory tract and lung.
SP-D preferentially interacts with the conserved core region of *Klebsiella* LPS containing mannose sequences in the O antigen. Thus, the mannose-containing O3 antigen allows binding with higher affinity than the O1, lacking these sugars.
To better understand the role of each of these constituents, we examined the infectivity and in vivo cytokine production of 4 noncapsulated different *K. pneumoniae* strains, two of which express O3 (K50/n, K55/n), and two express O1-antigen (K2/n, K21a/n).
An inverse correlation was observed between survival of *Klebsiella* in mouse lungs and cytokine production. O3 mannose containing serotypes, recognized by SP-D, triggered high IL-1β and IL-6 production but survived in significantly less amounts in the lung of infected mice as compared with O1 serotypes. These findings are also consistent with in vitro studies, showing that expression of IL-1β and IL-6 mRNA by human macrophages was significantly increased and selectively promoted by SP-D coated O3 *Klebsiella* serotypes. The results suggest that, survival of inhaled bacteria in the lung depends on their LPS structure and their interactions with innate immunity components. This is accompanied by release of pro-inflammatory cytokines, which may promote clearance of the pathogen from the lung. We might speculate that disbalance of host SP-D and therefore cytokine levels result in high susceptibility of the host to certain type of this pathogen.

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INTERFERON-γ UPREGULATES THE EXPRESSION OF PROINFLAMMATORY AND Th1 CYTOKINE mRNA IN CHICKEN HETEROPHILS DURING RECEPTOR MEDIATED PHAGOCYTOSIS

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The immediate response to invasive pathogens, clearance via the inflammatory response, and activation of appropriate acquired responses are all coordinated by innate host defenses. Recognition of microbes is accompanied by the induction of multiple cellular processes incuding the production of pro- and anti-inflammatory cytokines. Polymorphonuclear leukocytes (PMNs) are vital cellular components of innate response with the primary PMN in poultry being the heterophil. Priming is the potentiation of the phagocyte activation process by previous exposure to a priming agent. IFN-γ is a pleiotrophic cytokine involved in basically all phases of immune and inflammatory responses that has been shown to prime heterophil functional activities. In the present experiments, using real-time quantitative RT-PCR, we evaluated the role of recombinant chicken IFN-γ (rChIFN-γ) as a priming mediator to control heterophil responses at the level of gene transcription and expression of the mRNA for proinflammatory (IL-1β, IL-6, IL-8) and Th1 (IL-18 and IFN-γ) cytokine genes following stimulation with phagocytosis agonists opsonized and nonopsonized *Salmonella enteritidis*. rChIFN-γ primed the heterophils for an increase in transcription of pro-inflammatory cytokines induced by phagocytic agonists, but also upregulated expression of Th1 cytokine (IL-18 and IFN-γ) mRNA. Although rChIFN-γ priming modulated the expression of cytokine mRNA in heterophils stimulated by different phagocytic agonists, rChIFN-γ by itself did not directly induce gene expression of either proinflammatory or Th1 cytokines. The enhanced expression of cytokine mRNA does not appear to be differentially expressed depending on the receptor activated during phagocytosis. The results from the present experiments suggest that rChIFN-γ may play a significant role in avian innate immunity against *Salmonella* infection and may offer an adjunct use in the prevention and treatment of salmonellae infections in newly hatched chickens

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EFFECTS OF CHEMOKINE RECEPTOR CCR5 AND COMPLEMENT FACTOR C5A RECEPTOR IN PNEUMOCOCCAL PNEUMONIA AND GRAM-POSITIVE SEPSIS

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Streptococcus pneumoniae is the most common cause of community-acquired pneumonia and is associated with significant morbidity and mortality.

Phagocyte recruitment occurs rapidly during pneumococcal pneumonia in an attempt to control the bacteria. However, overzealous recruitment is associated with pathology. We have now utilised our models of pneumococcal pneumonia and sepsis to investigate the roles played by chemokine receptor CCR5 and complement C5a receptor (C5ar) in these responses.

CCR5-/- mice are significantly less susceptible to pneumococcal pneumonia than are control mice. This is associated with reduced pulmonary bacterial loads.

In contrast C5ar-/- mice are no more susceptible to pneumococcal pneumonia than are wild type mice. Histological staining of lung sections indicates that phagocyte recruitment is not impaired in these mice.

Following intravenous infection, CCR5-/- mice have significantly reduced levels of bacteraemia 24h post challenge although there is no difference in overall survival.

We are currently focusing on cytokine expression in infected CCR5-/- and C5ar-/- mice as well as the cell types mediating pathology via CCR5 during pneumococcal pneumonia.

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ROLE OF TLR4-MEDIATED IL-10 PRODUCTION IN REGULATORY T CELLS INDUCTION AND RESISTANCE TO INFECTION WITH *BORDETELLA PERTUSSIS*

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Signaling through Toll-like receptors (TLR) activates dendritic cell (DC) maturation and IL-12 production, which directs the induction of Th1 cells. We found that the production of IL-10, in addition to inflammatory cytokines and chemokines, was significantly reduced in DCs from TLR4-defective C3H/HeJ mice in response to *Bordetella pertussis*. TLR4 was also required for *B. pertussis* LPS-induced maturation of DCs, but other *B. pertussis* components stimulated DC maturation independently of TLR4. The course of *B. pertussis* infection was more severe in C3H/HeJ than in C3H/HeN mice. Surprisingly, antibody and antigen-specific IFN-γ responses were enhanced at the peak of infection, whereas antigen-specific IL-10 producing T cells were significantly reduced in C3H/HeJ mice. This was associated with enhanced inflammatory cytokine production, cellular infiltration and severe pathological changes in the lungs of TLR4-defective mice. Our findings suggest that TLR-4 signaling activates innate IL-10 production in response to *B. pertussis* which both directly, and by promoting the induction of IL-10-secreting type 1 regulatory T (Tr1) cells, may inhibit Th1 responses and limit inflammatory pathology in the lungs during infection with *B. pertussis*.

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SECRETORY LEUCOPROTEASE INHIBITOR IMPAIRS TOLL-LIKE RECEPTOR-MEDIATED RESPONSES

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Secretory Leucoprotease Inhibitor (SLPI) was originally identified as an inhibitor of neutrophil elastase (NE). More recently, an anti-inflammatory role has been described for SLPI that includes the ability to down regulate TNFα and matrix metalloprotease (MMP) expression in activated monocytes. SLPI's anti-NE activity is inhibited by oxidation; we postulated that oxidation might also inhibit its anti-inflammatory properties. We investigated the effect of oxidation and SLPI-NE complex formation on SLPI's anti-inflammatory activity in myelomonocytic U937 cells in response to lipopolysaccharide (LPS) and lipoteichoic acid (LTA), components of Gram(-) and (+) bacteria, respectively. LPS and LTA signal via TLR4 and TLR2, respectively and can activate NFκB in U937 cells. Our data shows that this occurs via pathways involving IRAK and IκBs. Recombinant SLPI can inhibit NFκB activation in response to both LPS and LTA. However, oxidised SLPI or SLPI-NE complexes are unable to attenuate LPS-induced NFκB activation. Oxidation of SLPI also inhibits its ability to down regulate LPS-induced TNFα gene transcription in U937 cells. Furthermore both IL-6 and MCP-1 protein production from LTA- and LPS-stimulated cells, respectively, is repressed by SLPI, an effect that is lost by oxidation. This data demonstrates that oxidation, and NE complex formation of SLPI diminishes its anti-inflammatory properties.

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INFLUENCE OF VARIOUS TYPE BACTERIAL LYPOPOLYSACCHARIDES ON HUMAN DENDRITIC CELL DIFFERENTIATION

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Dendritic cells (DC) begin to be initiated substantially into the clinical practice as anti-tumor vaccines. Maturation inductors added at DC incubation with tumor antigen greatly influence on the efficacy of such vaccines. Lypopolycaccharide does not only induce DC maturation, but a secretion of biologically active IL-12 activating NK, being of importance for the anti-tumor immunity. This work includes the studies on the influence of pharmaceutical preparation « Pyrogenal » and of high and low molecular fractions of *Shigella sonnei* LPC on the differentiation and activation of immature human DCs. Under the influence of LPC various types only insignificant part of the population (7-19%) became mature DCs of maximal ability for the antigen presentation. 25-64% of cells differentiated into macrophages playing an important role in anti-bacterial immune response. Cell activation was manifested in the induction and up-regulation of the expression level of co-stimulating molecules of CD40, CD80 and CD86. LPC of various types influenced the expression of co-stimulating molecules differently. The best inductor of co-stimulator expression and DC maturation was found to be a low molecular *Shigella sonnei* LPC. "Pyrogenal" absolutely did not induce CD80, though it significantly activated CD86 expression.

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THE ROLE OF IRF-8\ICSBP AND IRF-1 IN MACROPHAGE ACTIVITY

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Interferon regulatory factor 1 (IRF-1) and Interferon Consensus Sequence Binding Protein (ICSBP), also known as IRF-8 function as key elements in innate and adaptive immunity. As such, they are coactivators of various interferon-inducible genes including interferons themselves in macrophages. These IRF members are induced in these cells by pathogen infections that can be reproduced by IFN-γ and LPS treatment.

DNA microarray technology was employed to gain better insights into the involvement of these two transcription factors in the onset of the innate immune response and to identify their regulatory network in macrophages. Changes in the expression profile were analyzed in peritoneal macrophages of wild type mice and null mice to IRF-1 and ICSBP before and 4hrs after combined exposure to IFN-γ and LPS. Total RNA was then extracted and hybridized with Affymetrix Murine *Genome Array U74Av2*. The levels of RNA transcripts from treated macrophages in comparison to untreated were complied and compared. Microarray results were validated by RT-PCR analyses.

The expression pattern of 41 genes was significantly changed (up / down) in peritoneal macrophages extracted from wild type mice following treatment with IFN-γ and LPS while no changes were observed in samples of the same cells from IRF-1 and ICSBP null mice. These results suggest that both IRF-1 and ICSBP are involved in the transcriptional regulation of these genes. Furthermore, nine of the genes are related to immune response and inflammation.

We therefore suggest a broader role for IRF-1 and ICSBP in macrophages differentiation and maturation, being important inflammatory mediators.

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LIPOPOLYSACCHARIDE BINDING PROTEIN (LBP) DEFICIENT MICE HAVE AN IMPAIRED DEFENSE AGAINST GRAM-NEGATIVE BUT NOT GRAM-POSITIVE PNEUMONIA

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Background: LPS binding protein (LBP) facilitates binding of LPS from Gram-negative bacteria, and lipoteichoic acid and peptidoglycan from Gram-positive bacteria to the pattern recognition receptor CD14, leading to activation of immunocompetent cells. The role of LBP in host defense against localized bacterial infections such as pneumonia is unknown.

Aim and Methods: To determine the role of LBP in host defense against Gram-negative and Gram-positive pneumonia, LBP deficient (LBP-/-) and wild type (WT) mice were intranasally inoculated with 10^3 Colony Forming Units (CFU) *K. pneumoniae* or 10^5 CFU *S. pneumoniae*. Study endpoints were survival, bacterial outgrowth and host inflammatory responses. Statistics by Kaplan Meier and Mann-Whitney U test.

Results: In Gram-negative pneumonia, survival of LBP-/- mice was diminished compared to WT mice ($P < 0.05$). In contrast, in Gram-positive pneumonia, no difference in survival was seen. In *Klebsiella* pneumonia, lung bacterial outgrowth was enhanced in LBP-/- vs WT mice ($P < 0.05$). In pneumococcal pneumonia, bacterial outgrowth was similar in both mouse strains. In neither pneumonia model, a difference in granulocyte influx into the pulmonary compartment was seen.

Conclusion: LBP is important for an effective host defense against Gram-negative but not Gram-positive pneumonia.

INTERFERONS

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DE NOVO PROTEIN MIMETIC REPRESENTING THE ANTIVIRAL ACTIVITY OF INTERFERONS

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A major concern that emerged from clinical studies is that type I IFNs all generate a considerable number of undesirable, clinically observable, side effects. In addition, a variable proportion (1-40%) of patients treated with recombinant human (rh) IFN-α or IFN-β, especially rhIFN-α_2 and rhIFN-β Ser17, develop neutralizing antibodies to the IFN species used, that in some instances have been associated with clinical « resistance » to IFN. Besides, type I IFNs all generate fewer, chills, malaise, myalgia, headache, fatigue, and weight loss, and in certain cases these have been severe enough for treatment to be halted. These clinical observations make very important engineering of the type I IFNs mimetics specifically representing the antiviral and antiproliferative activities of the proteins, but avoid of most sites responsible for undesirable side effects. In the present work the *de novo* protein mimetic specifically representing the antiviral and antiproliferative activities of the type I IFNs was designed to avoid their undesirable side effects. The designed *de novo* protein demonstrates antiviral and antiproliferative activities, being almost as active as rhIFN-α_2. The protein reveals cytotoxicity only at concentrations exceeding minimal active concentration by 6 orders of magnitude.

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WITHDRAWN

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GLOBAL EFFECT OF PEG-INTERFERON-ALPHA ON GENE EXPRESSION IN PBMC IN VITRO

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The combination of interferon and ribavirin is the only known treatment for hepatitis C infection, but the mechanism of action is unknown. Utilizing oligonucleotide microarrays, we have examined the expression of 22,000 genes in human peripheral blood cells treated with these drugs. Treatment with ribavirin had very little effect on gene expression, however treatment with IFN-α had a dramatic effect, modulating the expression of approximately 1000 genes (at $P < 0.001$). In addition to genes previously reported to be induced by type I or type II interferons, many novel genes were found to be regulated, including transcription factors, a homeobox gene (HESX1), and an RNA editing enzyme apobec3. Chemokines CXCL10 and 11 were up regulated whereas CXCL5 was down regulated. Cytokines IL-15 and IL-18 were significantly induced whereas IL-1a and IL-1b were down regulated. Most cytokines were not affected by this treatment. TNF-alpha was only marginally enhanced although other genes associated with apoptosis, e.g. TRAIL, caspases etc. were highly induced. The results of the microarrays were confirmed by kinetic real time PCR. These data indicate that interferon treatment results in the upregulation of genes associated with the stress response, apoptosis, and signaling, while an equal number of genes are downregulated, including those associated with protein synthesis and other biosynthetic functions.

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SIMULTANEOUS BINDING OF TWO INTERFERON-α SPECIES TO SINGLE RECEPTOR SUBUNIT IFNAR2-EC

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Previously, we showed that two antigenically different human interferon alphas may bind to extracellular part of human interferon receptor (IFNAR2-EC) independently of each other. We observed that a second species of IFN (IFNα2c or hybrid CM-3) was capable of binding IFNAR2-EC coated plate, after the plate had been saturated with a different IFN species, and the second species was detectable with specific monoclonal antibody 1-36 (specific reaction with CM-3, epitope 63-85) or N-27 (specific reaction with IFNα2c, epitope 43-53). Our findings suggest that there is probably more than one IFN-binding site on the IFNAR2-EC molecule, and different IFN species might engage distinct parts of the interferon receptor for interaction. In order to investigate this hypothesis, we first determined the effect of incubation time on saturating IFNAR2-EC with IFN species by ELISA. It was established that after 3 hours, 15μg/ml of IFNα2c or CM-3 completely saturated 3μg/ml IFNAR2-EC. We used these conditions in competitive sandwich ELISAs to confirm the existence of another IFN-binding site on the IFNAR2-EC molecule, and showed that IFNAR2-EC can bind more than one interferon species. A combination of methods, including native electrophoresis complemented by Western Blot, chemical crosslinking followed by Western Blot, and size exclusion chromatography were used to demonstrate the formation of a heterotrimeric complex consisting of a single receptor subunit binding two different IFNα species.

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CSFV INFECTION OF VASCULAR ENDOTHELIAL CELLS INHIBITS ACTIVITY OF VIRALLY INDUCED IFN.

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Innate cellular response against viral infections involves production of IFN, activation of inflammatory pathways and triggering of cellular apoptosis. Viruses are generally potent inducers of IFN but many of them can block the synthesis and actions of IFNs. It is therefore crucial to understand the interaction of a virus with the host cell, and in particular the subversion of innate anti-viral responses arising from IFN production. Classical Swine Fever Virus (CSFV), a member of Pestiviruses, causes an acute haemorrhagic disease in pigs characterised by DIC, lymphopenia and thrombosis.CSFV can cause persistent infection in vascular endothelial cells. Endothelial cells therefore play an essential role in the pathogenesis of the disease. Primary vascular endothelial cells infected with CSFV do not secrete any active IFN. In this study we show evidence that CSFV is indeed able to block secretion of active IFN even in cells that have been treated with synthetic dsRNA or infected with a non related virus which can induce IFN. Data will also be presented about individual viral protein candidates able to prevent IFN production in cells infected with CSF virus.

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INTERFERON BETA 1A INHIBITS MONOCYTE-DERIVED DENDRITIC CELL MATURATION

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Dendritic cells are the most potent antigen presenting cell known, and are unique in their ability to initiate primary immune responses. They are thought to play a pivotal role in the decision between T cell activation and anergy. Interferon-β 1a (IFNβ) is used in the treatment of Multiple Sclerosis (MS), a common cause of neurological disability in young adults, characterised by multifocal CNS damage. Recently we have identified CD83 + DC in active MS plaque tissue [1], and have shown that IFNβ can interrupt the differentiation pathway of peripheral blood monocyte-derived DC [2]. To examine the mechanism by which IFNβ can block DC differentiation, we used human monocyte-derived DC. We have found that IFNβ can strongly block DC maturation by inhibiting toll-like receptor signalling pathways with a marked block in the activation of NFκB. The findings suggest that IFNβ therapy may work, at least in part, by dampening the DC immune response.

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INFLUENZA A, INFLUENZA B AND SENDAI VIRUSES DIFFERENTIALLY INFLUENZA AND SENDAI VIRUSES INDUCE A DIFFERENTIAL IFN, CHEMOKINE AND IKK-ε GENE EXPRESSION IN A549 LUNG EPITHELIAL CELLS

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Lung epithelial cells are the primary targets for respiratory viruses such as influenza A, influenza B and parainfluenza viruses. To study the ability of these viruses to induce host cytokine and chemokine gene expression we infected human lung epithelial A549 cells with influenza A (H1N1 and H3N2 strains), influenza B and Sendai viruses, isolated total cellular RNA and analyzed host cell cytokine and chemokine mRNA expression by Northern blotting and RT-PCR. Influenza A and B virus infection lead to a relatively weak induction of interferon (IFN-α, IFN-β) and IFN-like genes, IL-28 (IFN-λ2/3) and IL-29 (IFN-λ1). The expression of IRF-3 and IRF-7-activating kinase, IKK-ε was also weakly induced by influenza viruses. In similar experimental conditions Sendai virus infection activated the expression of IFN-α, IFN-β, IL-28, IL-29 and IKK-ε genes very well. Similarly, while Sendai virus readily induced TNF-α, CCL2 (MCP-1), CCL5 (RANTES), CXCL8 (IL-8) and CXCL10 (IP-10) gene expression, influenza A or influenza B virus-induced expression of these genes was either lacking (TNF-α) or occurred at a relatively low level. Pretreatment of A549 cells with IFN-α (100 IU/ml, 24 h) lead to a dramatic increase in influenza A virus-induced IFN, IL-28, IL-29 and chemokine gene expression. This induction correlated with enhanced expression of TLR-3, IKK-ε and IRF-7. The results indicate that in human lung epithelial cells influenza A and B viruses are relatively poor inducers of type I IFN, IL-28, IL-29 and chemokine genes, but their ability to induce these genes can be fully restored by pretreatment of the cells with type I IFNs.

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INDUCTION PATHWAYS FOR IFN-∀/∃EXPRESSION DURING VIRAL INFECTIONS OF MACROPHAGES

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Interferon (IFN)-∀/∃ is produced in response to viral infections, where this cytokine plays important roles in the first antiviral defence as well as the subsequent shaping of the immune response. We have investigated the virus-cell interactions and signal transduction pathways regulating IFN-∀/∃ expression during infections of murine macrophages with herpes simplex virus (HSV). We find that intracellular interactions between HSV and the macrophage are responsible for activating IFN-∀/∃ expression. This was concluded from the compromised IFN-∀/∃ induction by UV-inactivated virus and by a virus mutant unable to enter the cell. Using mutants deficient in each of the regulatory viral immediate-early genes we have found that ICP4 seems to play a role for induction of IFN-∀/∃ expression by HSV in macrophages. This is now being investigated more carefully. Moreover the mechanism for induction of IFN-∀/∃ was absolutely dependent on the NF-κB pathway and the dsRNA-activated protein kinase R (PKR), since macrophages expressing dominant negative forms of IκB kinase ∃ and PKR did not express IFN-∀/∃ upon infection with HSV. The importance of another central transcription factor, IRF-3, is under investigation as well as the involvement of Toll-like receptors. The result of these studies will be presented at the meeting.

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THERAPY OF CHILDREN WITH PYELONEPHRIT BY RECOMBINANT INTERFERON -ALPHA-2b WITH ANTIOXIDANTS

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63 children with acute pyelonephrit with a range between 1-14 years were under observation. Decrease ability to produce IFN-alpha and IFN-gamma correlated with increasing of lipid peroxidation (LPO) products and with increased lymphocyte quantity in blood were observed in all patients before treatment. Concentrations of tocopherol, ceruloplasmin, general antioxidant activity (AOA) in blood serum were determined. Calculated integral index (K) evidenced change in LPO-AOA balance for increasing of LPO. Control group included 21 childrens who received symptomatic therapy and suppositoria including oleum cacao without IFN-alpha-2b and antioxidants. Basic group included 42 children who received symptomatic therapy and recombinant interferon alpha-2b with antioxidants (tocopherol and ascorbic acid) in suppositoria (complex medicine Viferon). Viferon was prescribed in dose 150000-500000 IU of the IFN- alpha-2b per 1 suppositoria depending on the age; 1 suppositoria twice a day every 12 hours during 10 days. Viferon treatment increased indices of IFN-alpha and IFN-gamma, decreased lymphocyte quantity and indices of LPO, increased the content of tocopherol in blood serum and reduced the disbalance in LPO-AOA system (decreased « K » during treatment is positive prognosis). The use of Viferon promotes more quick liquidation symptoms of intoxication, clinical manifistation of acute pyelonephrit and bacteriouria.

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ACTIVATION OF IRF7 DURING PATHOGEN INFECTION: KINASES, PHOSPHORYLATION SITES, AND REGULATORY MECHANISMS

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Interferons (IFN) play a vital role in innate resistance to a wide variety of infectious agents. Interferon induction in response to pathogen invasion is tightly regulated. Much of this regulation is at the transcriptional level, and interferon regulatory factors (IRF), in particular IRF3 and IRF7, play an essential role in type I IFN regulation. Activation of IRF proteins provides both qualitative and quantitative control of IFN production through differential induction of distinct IFN isotypes.
IRF7 is activated in response to pathogen infection by phosphorylation on serine residues located in the carboxyl-terminal regulatory domain of the protein, and the non-conventional IkB kinases Ikk-ε and TBK1 have been implicated in this process. However, the exact sites of phosphorylation have yet to be definitively established, and the possibility of differential activation processes for distinct pathogens remains to be investigated.
We undertook a comprehensive approach of the identification of the phosphorylation sites of IRF7 in response to different inducing agents by examining mutant proteins in which specific serine residues were altered to alanine or aspartate. The phosphorylation patterns of these mutants were analyzed by 2D gels and their activity monitored by reporter assay. These patterns were compared to proteins phosphorylated in vivo in response to different pathogens or to individual kinases. Our data show that the serine-rich regulatory domain of IRF7 contains a kinase-recognition region that is necessary for distal phosphorylation but is not itself phosphorylated. The kinase recognition region is followed by multiple sites for phosphorylation, only some of which are needed for the majority of IRF7 transcriptional activity. In addition, the data suggest heterogeneity in the activation process by distinct pathogen activators.

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USE OF ANTIBODY MICROARRAY TO DETECT EXPRESSION OF SIGNAL TRANSDUCTION PROTEINS AFTER CELL TREATMENT WITH IFN-α CONSTRUCTS

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The object of present studies is the use of antibody microarray to determine what signaling proteins are either upregulated or downregulated as a result of treatment of Daudi cells with the various IFN-α's. For staining, avidin, biotin and peroxidase methodologies were used in conjunction with a Cataylzed Signal Amplification (CSA) system which is reported to be 50-fold more sensitive compared to conventional avidin-biotin reactions. The primary antibodies in all cases were polyclonal antibodies directed against the phosphorylated form of the signaling protein. In addition, these antibodies were specific to the particular amino acid phosphorylated (e.g. anti-pSTAT1 (Y701) and anti-pSTAT1 (S727). Preliminary assays show that pSTAT1 (Y701) was upregulated the most, followed by pSTAT2 (Y689) and pJak1 (Y1022/1023). Interestingly, pTyk2 (Y1054/1055) was essentially unaffected. This, however, could be due to the specificity of the antibody (phosphorylated at Y1054/1055) as it was noted that antibody directed against pSTAT1 (Y701) was upregulated almost 40 times greater than pSTAT1 (S727). This fact highlights the ability of these assays to not only determine the expression level of the proteins of interest, but to also demonstrate the point of phosphorylation of those proteins.

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EFFECTS OF IFN-α IN AICD OF HUMAN CD4⁺ T CELLS: A BALANCE BETWEEN INTRINSIC AND EXTRINSIC APOPTOTIC PATHWAYS

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Type I interferons (IFN-α/β) exert multiple effects on the immune system, affecting differentiation, proliferation and survival of different cell types, notably T cells. It was reported that IFN prevents cytokine-deprivation induced cell death of activated T cells without inducing their proliferation. We report herein the effect of IFN on early activation-induced cell death (AICD) using an *in vitro* differentiation model of human CD4⁺ T lymphocytes purified from cord blood. At 48 h post-activation, treatment of naïve T cells with IFN-α2 (before or at the moment of TCR/CD28 triggering) stabilizes mitochondrial transmembrane potential ($\Delta\psi_m$) and inhibits the aberrant exposure of phosphatidylserine (PS) residues on the outer plasma membrane leaflet, two of the early hallmarks of mitochondrial-dependent apoptosis. In addition, IFN-α increases the expression of the anti-apoptotic protein Bcl-2. Surprisingly, IFN-treated cells displayed higher levels of Fas receptor and an activated caspase pathway, as shown by caspase-8, -3 and PARP cleavage. Overall, these evidences suggest that IFN, produced in large amount by pathogen-activated DC, may induce in early activated T cells contrasting apoptotic signals inhibiting the intrinsic, mitochondrial-dependent pathway and activating the extrinsic caspase 8-mediated pathway.

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INTERFERON ALPHA-STIMULATED GENES RESPONSE IS ALTERED IN CHRONIC HEPATITIS C PATIENTS

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Type 1 interferon (IFN α/β) is expressed by most virus-infected cells and, via the jak-stat pathway, induces activation of hundreds of genes, many having critical anti-viral or immune functions. *In vitro* studies have recently suggested that HCV itself could inhibit this pathway, thus contributing to viral persistence.

Aim: Our aim was to assess expression of a panel of IFNα inducible genes potentially important for resolution of HCV-infection.

Methods: Eight chronically HCV-infected patients (genotype 1b) and 8 HCV RNA-negative controls were studied before antiviral treatment. Peripheral mononuclear cells (PBMCs) were cultured for 6 hours with IFNα at 20 and 100 IU/ml. After RNA extraction and cDNA synthesis, real-time quantitative PCR was carried out for a panel of genes including MXA, MXB, OAS-1, OAS-2, OAS-3, PKR, Rantes, MIG and IP10.

Results: In normal subjects, up-regulation was consistently observed after IFNα stimulation, with increases ranging from1.8 ± 0.7 fold- for Rantes to 31.3 ± 25.8 fold for PKR. A clear dose effect was demonstrated between 20 and 100 IU/ml, resulting in an average of 2-fold greater expression of each gene with the higher dose. Three genes, MXA, OAS-2 and Rantes were more comprehensively studied in the 16 subjects. Significant correlation was observed between MXA and OAS2 gene expression (20UI: $r = 0.73$, $p < 0.04$). After stimulation with both 20 and 100, gene expression, levels were significantly lower in HCV-positive compared to controls (OAS2 20UI: 6 vs 12, 100 IU: 10 vs 24, $p < 0.01$; MXA 20UI: 8 vs 17, 100 IU: 11 vs 34, $p < 0.01$; Rantes 20IU: 0.5 vs 1.4, 100IU: 0.5 vs 2.1, $p < 0.05$). Absence of global cellular dysfunction by HCV infected PBMCs was demonstrated by successful IL2 and IFNγ production post-stimulation with PMA and ionomycin.

Conclusion: Our results show that IFNα gene responses are impaired in PBMCs from HCV-infected patients. The cell populations most susceptible to viral inhibition of the IFNα pathway remain to be determined.

KNOCKOUTS

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INTERLEUKIN 18 IMPROVES THE EARLY ANTIMICROBIAL HOST RESPONSE TO ESCHERICHIA COLI PERITONITIS

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Background: IL-18 plays an important role in the host response to lipopolysaccharide (LPS).
Aim and Methods: To determine the role of endogenous IL-18 during peritonitis, IL-18 gene-deficient mice (KO) and wild-type mice (WT) were i.p. infected with *E. coli* (10^4 CFU). Data are means ± SE. Statistics by Mann Whitney test ($^*p < 0.05$).
Results: Peritonitis caused a dose dependent increase in IL-18 concentrations in peritoneal fluid (PLF) and blood. After infection, KO mice demonstrated an impaired antibacterial defense compared to WT mice as indicated by an enhanced bacterial outgrowth: PLF: KO: 53.4 ± 5.7 vs. WT: 23.6 ± 4.4*; blood: 3.9 ± 0.8 vs. 1.7 ± 0.8* (all × 10^8 CFU/ml). The relative inability of KO mice to clear *E. coli* was not due to an intrinsic defect in the phagocytosing capacity. KO mice displayed an increased neutrophil influx into the peritoneal cavity (KO: 5.2 ± 0.9 vs. WT: 2.2 ± 0.2*; all × 10^6 cells/ml) but these migrated neutrophils were less activated as reflected by a reduced CD11b surface expression: PLF: KO: 600.8 ± 51.6 vs. WT: 741.3 ± 42.4* (MCF).
Conclusion: These data suggest that endogenous IL-18 plays an important role in the early antibacterial host response during *E. coli* induced peritonitis.

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EVIDENCE FOR GENETIC MODIFIERS OF SUSCEPTIBILITY TO ARTHRITIS' AORTITIS AND CUTANEOUS INFLAMMATION IN IL-1 RECEPTOR ANTAGONIST-DEFICIENT MICE

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IL-1ra-deficient Balb/c develop aortitis, arthritis and cutaneous inflammation, C57BL/6 do not. To assess the genetic contributions from the background of Balb/c to these inflammatory diseases, we have crossed two strains to generate IL-1ra-deficient C57BL/6 × Balb/c F1 and F2. At 200 days we archived 182 F2 and 12 F1 cadavers, and their DNA. These have been compared with 27 C57BL/6 and 18 Balb/c. Arthritis, preliminarily ascertained from joint swelling, was seen in 20/27 Balb/c mice, but not in the F1. It re-emerged in 10/182 F2 animals. Arthritis in the F2 and could be explained by a requirement for homozygosity of recessive alleles at two independent loci. Cutaneous inflammation was not observed in 12 F1 and 95 F2, by ear thickness measurement. Aortitis was frequent (6/14), in a random sample of the F2 and present (2/12) in the F1. These results suggest a suppressive effect on aortitis of a number of C57BL/6 loci, but clearly they are not simply dominant. The distribution of the three diseases among these and other crosses demonstrates that there are different genetic susceptibility determinants for each disease. The variation that we have seen within strains and within the F1 demonstrates the significance of environmental factors.

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TRANSGENIC MICE OVEREXPRESSING A NOVEL CYTOKINE (IL-31) DEVELOP A SEVERE PRURITIC SKIN PHENOTYPE RESEMBLING ATOPIC DERMATITIS

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We have cloned a novel four-helix bundle cytokine, designated *IL-31*, which binds and signals through a heterodimeric receptor composed of IL-31RA and oncostatin M-receptor β (OSM-Rβ). The IL-31 receptor belongs to the G-CSF/IL-6/gp130 receptor family. To evaluate the in vivo effects of IL-31 overexpression, we generated multiple founders of transgenic (Tg) mice expressing the murine form of the gene, driven by the lymphocyte-specific promoter/enhancer Eμ/lck, or the ubiquitous promoter, EF1α. The IL-31 Tg mice develop a skin phenotype around 4-8 weeks of age, consisting of piloerection and mild to severe alopecia. The Tg skin is also pruritic, as evidenced by the scratching behavior of the mice, often excessive enough to induce excoriation and lesions of the skin. Histopathology of lesional Tg skin has revealed many alterations, including hyperkeratosis, acanthosis, inflammatory cell infiltration, and an increase in mast cells. The Tg skin phenotype was recapitulated in IL-31 Tg → RAG-$1^{-/-}$ radiation bone marrow chimeras, and with chronic delivery of purified IL-31 protein to adult C57Bl/6 or Balb/c mice, demonstrating that it is not due to a developmental defect. The phenotype of the IL-31 Tg mice strongly resembles that of atopic dermatitis (AD) patients, and mouse models of AD. AD is a common chronic inflammatory disease that is characterized by hyperactivated cytokines of the helper T cell subset 2 (Th2). IL-31 is preferentially expressed by Th2 vs. Th1 cells, and its receptor is expressed by many types of epithelial cells (in skin, lung, etc). Thus, IL-31 may contribute to the pathogenesis of allergic diseases such as AD and asthma. An antagonist of IL-31 might therefore represent a viable therapeutic for these and other indications.

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CHARACTERIZATION OF THE ROLE OF CCR5 IN LEUKOCYTE MIGRATION AND HOST DEFENCE DURING A GENERALIZED HERPES SIMPLEX TYPE 2 INFECTION IN MICE

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The CC chemokine receptor CCR5 is an important co receptor for human immunodeficiency virus and is therefore a major hope for developing anti-CCR5-based therapies for HIV-1. However, it is not known whether CCR5 is critical for normal antiviral T-cell response. Our objective is to describe the role of CCR5 in the response during a generalized herpes simplex type 2 infection. After intra-peritoneal infection with HSV-2 in 3-4-week-old mice higher viral titers were seen in the liver and the brain of CCR5-defient mice compared to wild type littermates. In particular we noted strongly impaired ability to control virus replication in CCR5-/- mice examined late during the course of the infection. We also found that despite no differences in the size of the spleen on day 0, on day 6 spleens from CCR5-deficient mice were significantly smaller than spleens from wild type mice. In ongoing experiments we are evaluating the mechanism behind this observation as well as the functional consequences with respect to antiviral defence. The results of these studies will be presented at the meeting.

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TNF PROTECTS FROM CHOLESTEROL-DIET INDUCED TARGET-ORGAN-DAMAGES, PROMINENTLY FROM LIVER STEATOSIS

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The Tumour Necrosis Factor (TNF) blocking therapy with antibodies is successfully applied in patients with rheumatoid arthritis, but might affect the fat-triglyceride homeostasis. As a respective model, high cholesterol diet (1.25%), a risk factor for dyslipidemia was investigated in TNF gene deficient (ko) mice for target-organ-damages. We found a dramatic increase in the liver-weight in ko-mice of 170% compared to 50% in wt-mice. The liver enlargement was associated with hepatocyte swelling and a diffuse lipid vascularization of intracellular lipids. The steatotic liver was composed of saturated and unsaturated (prominently mono- but also polyunsaturated) fatty acids, as assessed by the trans-esterification glyceride analysis using gas chromatography. No signs of inflammation and liver steatitis were observed. The Scavenger Receptor (SR)-BI and the insulin's postreceptor signals, two molecular mediators of lipid turnover and disposal, were significantly decreased. In contrast, Cyclin D1 levels were maintained.
Furthermore, already ko-mice with normal diet show increased levels of Cyclin D1 and a diminished content of liver-fat, compared to wt-mice. In conclusion, we show that TNF ko-mice have an abnormal metabolic phenotype' in particular upon a high lipid-load. Therefore careful checking and monitoring of the lipid homeostasis in patients treated with TNF neutralizing therapies is proposed.

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CAUSAL ROLE OF TNF IN LPS-INDUCED HYPOTENSION BUT NOT TACHYCARDIA

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TNF is a critical mediator of sepsis including cardiovascular complications. We investigated hemodynamics parameters in freely moving TNF gene knockout (ko) mice in a D-Galactosamine sensitised LPS model using radio-telemetry. Both mice, wild type (wt) and TNF-ko mice, showed increases in the heart rate upon LPS treatment from 450 beats per minutes (bpm) to 650 bpm. In the ko-mice this increase persisted for 12 to 15 hours with normal blood pressure values, whereas wt-mice developed a fatal hemodynamic collapse after 5-6 hours of LPS treatment. Ko-mice regained their circadian rhythm and physical activity within 24 hours.

In wt-mice, the liver was hemorrhagic and the apparatus of protein synthesis was impaired, as estimated by protein complex formation of the translation initiator eIF-4E/G and ribosomal protein S6. In contrast, the inducible Nitric Oxide Synthase (iNOS) was strongly enhanced.

In the ko-mice the apparatus of protein synthesis was maintained and despite the absence of TNF, iNOS was induced to the same extend as in wt-mice. The iNOS upregulation was hence not sufficient for causing the hypotension and hemodynamic collapse in sepsis, whilst the shock was rather associated with defects in the protein-apparatus in vital organs such as the liver.

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THE CHOLINERGIC NERVOUS SYSTEM IMPAIRS HOST DEFENSE DURING ABDOMINAL SEPSIS

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The nervous system, through the vagus nerve, can downregulate inflammation by decreasing the production of TNF-α by macrophages during experimental endotoxemia.
To evaluate the role of this "cholinergic anti-inflammatory pathway" during septic peritonitis mice received an intraperitoneal injection with $5*10^4$ CFU *E.coli*. Peritonitis was preceded by either inhibition of the cholinergic anti-inflammatory pathway by unilateral cervical vagotomy or by stimulation of this pathway by oral pretreatment with nicotine.
Previous cervical vagotomy resulted in an enhanced influx of neutrophils into the peritoneal cavity, higher levels of proinflammatory cytokines in peritoneal lavage fluid (PLF) and a marked increase in liver necrosis in response to peritonitis. Nicotine pretreatment strongly decreased bacterial clearance, cell influx and PLF proinflammatory cytokine levels; in addition, nicotine attenuated liver necrosis and reduced survival.
During septic peritonitis, inhibition of the cholinergic anti-inflammatory pathway induces a proinflammatory state whereas stimulation of this pathway strongly impairs host defense, at least in part by inhibition of the production of proinflammatory cytokines. These data provide the first evidence for an important role of the cholinergic nervous system in regulating host defense during infection.

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A STRUCTURAL VARIANT OF THE IL-1 RECEPTOR ACCESSORY PROTEIN (AcP) IS EXPRESSED PRIMARILY BY NEURONS

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We have discovered a splice variant of the IL-1R Accessory protein (AcP) mRNA that encodes a novel form of the receptor. The isoform, which we have termed AcPb, arises via splicing of an alternative exon at the 3' end of the transcript. This exon contains features of the TIR domain, a motif conserved in AcP and other members of the IL-1R and TLR families, as well as approximately 100 additional amino acids with no obvious sequence homology or putative function. Expression analysis has revealed that whereas AcP is widely expressed in many tissues and cell types, the AcPb transcript is found at significant levels only in the central nervous system. In situ hybridization as well as immunohistochemical analyses suggest expression may be restricted to neuronal cells throughout the brain. The extracellular and transmembrane portions of AcPb are identical to those of "traditional" AcP, which suggests that AcPb may be recruited to ligand-bound IL-1R. If this interaction occurs, however, it does not appear to lead to activation of traditional IL-1-related pathways including NF-kB. Data will be presented from experiments designed to elucidate the functional role of this novel receptor.

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THE PRESENCE OF INTERLEUKIN-4 (IL-4) RECEPTOR IN RAT HIPPOCAMPUS PERMITS A PROTECTIVE ACTION OF IL-4 ON LPS-INDUCED NEURODEGENERATIVE EFFECTS

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Interleukin-4 (IL-4), which is released from T helper type 2 cells and mast cells exhibits anti-inflammatory properties which are mediated by activation of its receptor (IL-4R). Lipopolysaccharide (LPS) administration stimulates immune activation and inflammation; in hippocampal cells LPS leads to deterioration in cell function. Our data demonstrate the presence of IL-4R on neuronal membranes in rat hippocampus, and that in hippocampus, as in other tissues, IL-4 induces an intracellular signalling cascade by increasing phosphorylation of Janus tyrosine kinase-1 (JAK1) which subsequently acts as a catalyst to phosphorylate signal transduction and activator of transcription-6 (STAT6). We report that IL-4 administration to rats significantly abrogated LPS-induced activation of c-jun-N-terminal kinase (JNK), c-jun and cytochrome c in hippocampus. Significantly, we observed that IL-4 induced a concomitant increase in the hippocampal concentration of interleukin-10 (IL-10). These data suggest that treatment with IL-4 confers a protective effect in the brain by suppressing potentially detrimental effects of LPS, and that this protection may be related to an enhanced release of IL-10.
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LIPOPOLYSACCHARIDE-INDUCED INCREASE IN SIGNALING IN HIPPOCAMPUS IS ABROGATED BY IL-10 – A ROLE FOR IL-1β?

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Parenterally-administered lipopolysaccharide (LPS) induces an increase in the concentration of interleukin-1β (IL-1β) in the rat hippocampus and this effect plays a significant role in inhibiting long-term potentiation (LTP). Recent reports indicate that the anti-inflammatory cytokine, IL-10, antagonizes certain effects of IL-1β in hippocampal tissue and therefore, if the effects of LPS are mediated through an increase in IL-1β, it might be predicted that IL-10 would also abrogate the effect of LPS. Here we report that IL-10 reversed the inhibitory effect of LPS on LTP and the data couple this with an inhibitory effect on the LPS-induced increase in IL-1β mRNA and protein in hippocampus. LPS treatment increased hippocampal expression of IL-1 receptor Type I and there was evidence of enhanced downstream phosphorylation of IRAK and the stress-activated kinases, JNK and p38; these LPS-induced changes were reversed by IL-10, which concurs with the idea that these events are triggered by increased activation of IL-1RI by IL-1β. We provide evidence which indicates that LPS treatment leads to evidence of cell death and these changes were reversed in LPS-treated rats which received IL-10. The evidence is therefore consistent with the idea that IL-10 acts to protect neuronal tissue from the detrimental effects induced by LPS.

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THE RESTRAINT STRESS-INDUCED ELEVATION IN PLASMA INTERLEUKIN-18 in MICE. A SPECIAL REFERENCE TO AN ACTION OF CASPASE-1

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Many studies demonstrate that physiological or psychological stressors can alter immune functions. Interleukin-18, a proinflammatory cytokine, is characterized by pleiotropy and redundancy of action. We reported elevated higher plasma IL-18 levels in drug-naive patients with depression and anxiety disorder. In the present study, in order to confirm whether or not the phenomenon is attributed to a stress-response, mice were subjected to 6-hours restraint and found the elevation of plasma IL-18. Furthermore, to elucidate the mechanism whereby stress stimuli trigger the production of Il-18, the involvement of free radical was investigated. Plasma IL-18 elevation was not found in wild type mice administered with free radical scavenger nor caspase-1 knockout mice subjected to the stress paradigm. These results indicate that free radical may contribute to the activation of caspese-1 causing cleavage of pro IL-18 into IL-18 excretion during stress condition.

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TNFα- AND MCP-1 mRNA DURING WALLERIAN DENEGENERATION IN THE PERIPHERAL AND CENTRAL NERVOUS SYSTEMS

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Peripheral nerve injury results in a considerable accumulation of macrophages, which is believed generally to contribute to nerve regeneration. Dorsal spinal root injury likewise causes rapid degeneration of dorsal column axons but elicits a weak and delayed inflammatory response. The selective monocyte infiltration into injured peripheral nerves is mediated at least in part by MCP-1 (monocyte chemoattractant protein-1), which in turn is induced in part by tumour necrosis factor -α (TNF-α) (Subang and Richardson 2001).
Tissues were harvested 6hours, 1day, 4 days after the sciatic nerve transection for peripheral nerve injury model and 1day, 3days and 9 days following dorsal rhizotomy in central nerve injury model. TNF-α and MCP-1 mRNA in peripheral nerve and central nerve injury models were quantified using real-time PCR (Taqman®chemistry). 18s rRNA was used as internal standard.
Changes in TNF-α mRNA were modest, not significantly different as compared to contra-lateral side and from those after sham injury and do not account for the significant increase in MCP-1 mRNA levels in distal degenerating peripheral nerve. The induction of MCP-1 mRNA seen during Wallerian degeneration in peripheral nerves were not seen during Wallerian degeneration in the spinal cord.

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WIDESPREAD INDUCTION OF ENDOGENOUS IL-1 IN THE RAT BRAIN AFTER INTRASTRIATAL ADMINISTRATION OF EXOGENOUS IL-1

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Injection of IL-1 in the rat brain exacerbates ischaemic and excitotoxic brain damage. IL-1 worsens excitotoxic injury when administered in a number of brain sites. To indicate possible site(s) of action we studied the distribution of exogenous and endogenous IL-1 after striatal injection of human (h)IL-1β.
Rats were anaesthetised and hIL-1β (10ng) injected into the striatum. After 2h or 6h animals were sacrificed, CSF taken and different brain regions dissected free. IL-1β levels were measured using species-specific ELISA, enabling us to distinguish between hIL-1β and endogenous rat (r)IL-1β.
Highest amounts of hIL-1β were seen at 2h and 6h in the striatum and CSF, with minor diffusion to other brain regions. There was little induction of endogenous IL-1β by 2h, apart from a slight increase in the meninges. However, significant increases in rIL-1β were observed by 6h in many regions, the most marked being in the CSF.
These data suggest that exogenous IL-1 gains access to the CSF in relatively high amounts even when given directly into brain tissue. Once in the CSF it acts to increase endogenous IL-1 production, mainly in choroid plexus or meningeal macrophages, after which it could act as a volume transmission signal.

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NEUROPROTECTION BY A NON-ERYTHROPOIETIC DERIVATIVE OF ERYTHROPOIETIN

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Erythropoietin (EPO) has neuroprotective activities in vitro, in various models of neuronal apoptosis. In vivo, we showed that EPO can be administered systemically and crosses the blood brain barrier, resulting in protective effects in animal models of cerebral ischemia, brain trauma, spinal cord injury, and experimental autoimmune encephalomyelitis. However, EPO also increases the hematocrit in models were it is administered chronically.
To circumvent the undesirable effects of an increased hematocrit, we have developed EPO derivatives that have lost the effect on the hematocrit. One of these retain activity in models of neuronal injury in vivo and in vitro.
The neuroprotective action of these molecules in models of brain injury is associated with decreased inflammation and inhibition of inflammatory cytokines. When tested in acute and chronic models of EAE and experimental diabetic neuropathy, EPO proved to be protective indicating its usefulness in inflammatory diseases of the nervous system.
As EPO also prevented cardiomyocyte death in a model of acute myocardial infarction in vivo, we think that these molecules have tissue protective actions also outside the nervous system. The data with non-eryhropoietic EPO derivatives clearly show that these effects are independent on the effect on erythropoiesis.

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SYSTEMIC ADMINISTRATION WITH THE IL-8 INHIBITOR REPERTAXIN REDUCES NEUTROPHIL (PMN) INFILTRATION AND TISSUE DAMAGE IN CEREBRAL ISCHEMIA-REPERFUSION INJURY

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Infiltration of PMN is thought to play a role in brain ischemic damage. We studied the effect of repertaxin, a small molecule inhibitor of IL-8, on PMN infiltration and tissue damage (necrosis volume) in cerebral ischemia in rats. Ischemia was induced by middle cerebral artery occlusion (MCAO) in rats and PMN infiltration and brain damage were evaluated 24h later. In one model the MCA was permanently ligated while in a second model 90 min MCA ischemia was followed by reperfusion. Repertaxin was administered intravenously at the time of ischemia and subcutaneously every two hours for four times at the dose of 15 mg/kg. In the permanent MCAO, repertaxin inhibited PMN infiltration, evaluated as myeloperoxidase activity in the brain cortex homogenate (untreated: 0.305 ± 0.029 DeltaA/min/mg protein; repertaxin 0.191 ± 0.022; P<0.01); however, in this model repertaxin did not decrease tissue damage, evaluated as necrosis volume by tetrazolium stain (necrosis volume was 146 ± 14 mm3 in untreated and 126 ± 19 mm3 in repertaxin group). In the ischemia-reperfusion model, repertaxin inhibited PMN infiltration (untreated: 0.334 ± 0.041 DeltaA/min/mg protein; repertaxin 0.153 ± 0.040; P<0.01) and also protected from tissue damage by decreasing by 44% the necrosis volume from 265 ± 24 mm3 to 149 ± 27 mm3 (P<0.01). Since the extent of PMN infiltrate, and inhibition by repertaxin, was comparable in the two models (parmanent and reversible MCAO) we conclude that reperfusion induces PMN activation and in this model inhibition of IL-8 by repertaxin can be of pharmacological interest.

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ACUTE STRESS PROMOTES AN IMMUNOSUPPRESSIVE/ANTI-INFLAMMATORY CYTOKINE PHENOTYPE IN RATS

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We examined the possibility that increased production of the anti-inflammatory cytokine IL-10 mediates the suppression of pro-inflammatory cytokines such as TNF-α observed following exposure to acute stress. The results demonstrate that exposure of rats to 15 min of swim stress increased (\\ 126\\3 fold) lipopolysaccharide (LPS; 100 ug/kg;i.p.)-induced IL-10 production, and profoundly suppressed LPS-induced TNF-α production, thus promoting an immunosuppressive cytokine phenotype.
In contrast, stress failed to significantly alter IL-10 or TNF-α production in the absence of stimulation with LPS. Following immunoneutralisation of IL-10 in LPS-treated rats, the stress-induced suppression of TNF-α was still evident, indicating that it occurred independently of increased IL-10 production. As stress activates the sympathetic nervous system, we examined the role of catecholamines in promoting the immunosuppressive cytokine phenotype. Pre-treatment with the peripherally acting β-adrenoceptor antagonist nadolol (0.2 mg/kg; i.p.) completely blocked the increase in IL-10, but failed to alter the suppression of TNF-α induced by stress, further demonstrating a disassociation between the ability of stress to increase IL-10 and suppress TNF-α production. In conclusion, acute swim stress increases production of the anti-inflammatory cytokine IL-10, but this does not mediate the suppression of TNF-α induced by stress. Moreover, catecholamines mediate the increase in IL-10, but not the suppression of TNF-α induced by acute stress. Thus further studies are required in order to elucidate the mechanism by which acute stress suppresses LPS-induced TNF-α production.
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EVALUATION OF THE ROLE OF ONCOSTATIN M EXPRESSION IN MULTIPLE SCLEROSIS

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Oncostatin M (OSM) is a potent modulator of inflammation. In this study we examined the presence and putative role of OSM during neuro-inflammation. OSM levels in cerebrospinal fluid collected from patients with multiple sclerosis (MS) was raised compared to controls and correlated with the clinical severity of the disease (OSM was undetectable in the serum). Previously we have shown that OSM is involved in modulation of leukocyte recruitment. To provide an incite into the role of OSM we employed an 'in house' human blood-brain barrier model (co-culture of the endothelial-like cell line ECV304 and the astrocytoma line 1321NI) to examine the effect of OSM on cytokine expression. Stimulation of the astrocytic cells only (brain side) of the co-culture with OSM (0.001-10ng/ml) showed a dose-dependent increase in IL-6 and MCP-1 expression from the endothelial cells. This was not due to OSM leakage through the barrier model but indicted signaling between the cell types. These results demonstrate that OSM levels correlate with the clinical severity of MS and expression within the central nervous system may be involved in generating the chemotractic gradient required for the leukocyte recruitment, from the peripheral circulation into the brain, in neuro-inflammation.

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INDUCTION OF INFLAMMATORY RESPONSES IN BRAIN FOLLOWING INTRANASALLY-DELIVERED CHOLERA TOXIN: IMPLICATIONS FOR AB TOXINS AS ADJUVANTS IN NASAL VACCINES

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Intranasally-delivered vaccines provide an attractive alternative to conventional, parenterally-delivered vaccines by eliminating the need for injections and stimulating mucosal immunity. However, they require the inclusion of adjuvants, which may be associated with an increased risk of neurological reactions due to transport of vaccine components to the brain via the olfactory system. Here, we assess the consequences in the brain of intranasally delivered cholera toxin (CT), a powerful mucosal adjuvant and AB toxin. An increase in mRNA expression for cyclooxygenase-2 (COX-2) was observed in the hypothalamus of mice immunized intranasally with CT. COX-2 catalyzes the formation of prostaglandins in the brain and plays a role in fever induction. IL-1β and MCP-1 mRNA levels were also increased in the murine hypothalamus following CT treatment. In contrast, CT had no effect in the hypothalamus when administered parenterally, whereas LPS delivered parenterally induced fever via induction of IL-1β in the hypothalamus. This may suggest that CT cannot cross the blood-brain barrier (BBB) when given systemically, but that it can use the olfactory nerve to bypass the BBB and target the brain when given intranasally. These factors may have implications for the use of AB toxins as adjuvants in nasally delivered vaccines.

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IL-1β SIGNALLING IN THE BRAIN IN THE ABSENCE OF IL-1R1

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Interleukin-1 (IL-1) is a pro-inflammatory cytokine whose involvement in neuronal damage following acute neurodegeneration is well established. IL-1 binds to the type-1 receptor (IL-1R1), which is the only known functional receptor for IL-1. An accessory protein (IL-1AcP) is then recruited, leading to signal transduction. However, recent evidence suggests that some actions of IL-1 in the brain may be independent of IL-1R1, pointing to a new, yet unknown functional receptor for IL-1. The objective of the present study was to compare the signalling mechanisms responsible for the actions of IL-1 in primary mixed glia from wild-type and IL-1R1 deficient (IL-1R1$^{-/-}$) mice, and to identify IL-1R1-independent IL-1 actions.
Our previous results have shown that IL-1β activates mitogen-activated protein kinases (MAPKs) and nuclear factor-κB, and induces the release of IL-6 and PGE_2, in glia isolated from wild-type, but not IL-1R1$^{-/-}$ mice. However, DNA microarray studies, and subsequent semi-quantitative RT-PCR and Northern blot analysis, suggests the existence of IL-1R1-independent IL-1 signalling pathways. The identity and nature of a novel IL-1 receptor in the brain that mediates these effects remains unknown.

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INFLAMMATORY SIGNALS FROM PERIPHERY TO THE BRAIN

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Interleukin (IL)-1 and Tumor necrosis factor-a (TNF-α) is a major mediator of inflammation and exerts pleiotropic effects on the neuro-immuno-endocrine system. To evaluate the role for IL-1 and TNF-α in stress response, we made use of IL-1α/β and TNF-α triple-deficient mice (IL-1/TNF KO) along with IL-1α/β double-deficient mice (IL-1 KO), and TNF-α-deficient mice (TNF KO). Peripheral administration of recombinant murine IL-1-α (rmIL-1-α), but not recombinant murine TNF-α, triggered a febrile response and anorexia in IL-1/TNF KO mice, while central administration of rmTNF-α did trigger a febrile response and anorexia. We further analyzed the molecular mechanism for the difference between peripheral IL-1- and TNF-α-induced stress response, and demonstrated that the induction of IL-6 in the brain is differently regulated in response to IL-1 and TNF-α, while both cytokines induced cox-2. Central injection of PGE2 induced transient febrile response in IL-6-deficient mice, and the treatment did not induced IL-6 transcription. Our results indicated that the induction of cox-2 is not sufficient for prolonged induction of fever response, and IL-6 plays an important role in enhancing the febrile response downstream of PGE2.

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INVOLVEMENT OF IL-1 SYSTEM IN APOPTOSIS OF MURINE HIPPOCAMPAL GRANULE NEURONS INDUCED BY TRIMETHYLTIN INJECTION

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The dentate gyrus granule neurons of the hippocampus are well recognized as relatively resistant to injury induced by pathogenic stimuli. In our studies we applied the rare model of selective degeneration of murine hippocampal granule cells, caused by trimethyltin (TMT) administration in dose 2.5 mg/kg b.w.. Granule cell death bore the apoptotic features: chromatin condensation, oligonucleosomal DNA fragmentation and caspase-3 activation, evident as early as 1 day after intoxication. Using RPA method we found the increase of interleukin-1beta (IL-1beta) and IL-1 receptor antagonist (IL-1ra) mRNA in the hippocampus 3 days after treatment, confirmed on the protein level using Western blot method. Immunocytochemical studies showed that the cellular source of both cytokines was ameboid microglia, activated in the region of degeneration. However, the distribution of IL-1beta and IL-1ra was distinct within the granule layer. Whereas IL-1beta-positive cells were mainly localized in the crest of dentate gyrus, where the highest number of apoptotic neurons appeared, IL-1ra-positive microglia was practically restricted to the remaining dentate regions. Temporal and spatial relation between apoptosis and expression of IL-1 family proteins suggests the active involvement of IL-1beta in neuronal apoptosis, whereas IL-1ra could be engaged in protection of spared granule cells.

NOVEL CYTOKINES

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NAMING NOVEL CYTOKINES

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The HUGO Gene Nomenclature Committee (HGNC) has to date provided unique gene symbols for over half of the estimated 25,000 human genes. Standardised gene nomenclature is an essential resource for all scientists and gene names describing structure, function or homology are preferred. However, we have found that this has raised concerns with the immunological community, as assigning gene names based on sequence similarity may imply function. Therefore, in conjunction with the nomenclature committees of the International Immunological Society (IUIS) and the International Society for Interferon and Cytokine Research (ISICR) we have agreed a set of guidelines that we can all use. Here, we will present these guidelines and provide examples of confusion that have occurred in the literature when approved nomenclature is not used.

Ideally, we like to provide approved gene symbols before manuscript submission; though naturally any pre-publication information obtained by us whilst doing so remains confidential. This avoids any possible conflicts with existing symbols and also ensures that the gene is promptly included in our database.

The HUGO Gene Nomenclature Committee webpage can be found at URL http://www.gene.ucl.ac.uk/nomenclature/ and we can be contacted via email: nome@galton.ucl.ac.uk

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IMMUNOREGULATORY PROPERTIES OF IL-4 AND IL-4δ2 SYSTEM

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Cytokine isoforms resulting from alternative splicing of correspondent mRNA were the products of the same gene but they had different and in some cases opposite influence on target cells and could change cell reactions they mediate. The alternative splice variant of IL-4 in which the second of four exons (residues 22-37) was omitted, had been designated as IL-4δ2. IL-4 regulatory function as a cytokine was performed via cooperative two-centered specific interaction with heterodimer receptor IL-4R, characterized by high affinity interaction (K_{d1}) with α chain and some weaker interaction (K_{d2}) with the receptor γ chain. Specific interaction of IL-4 with the receptors of thymocytes and human thymic epithelial cells (VTEC2.HS) was characterized by $K_{d1} = (0.5 \pm 0.09) \times 10^{-10}$ M, $K_{d2} = (2.5 \pm 0.2) \times 10^{-9}$ M and $K_{d1} = (0.87 \pm 0.4) \times 10^{-10}$ M, $K_{d2} = (4.5 \pm 0.9) \times 10^{-9}$ M, respectively. IL-4δ2 interacted specifically only with α chain of IL-4R on these same cells and showed $K_d = (2.7 \pm 0.3) \times 10^{-10}$ M and $K_d = (3.3 \pm 0.3) \times 10^{-10}$ M, respectively. IL-4 and IL-4δ2 effectively compete for the common site on the α chain of the receptors. IL-4 stimulated mitogen-induced thymocyte proliferation. IL-4δ2 acted as its antagonist and caused inhibition of thymic lymphoid cell proliferation. At the same time, IL-4δ2 potentiated proliferation of thymocytes stimulated by IL-2. IL-4δ2 inhibited IL-4-induced synthesis of IgE. IL-4 stimulated proliferative response of TEC preliminary incubated with mitogen. The co-mitogenic effect of IL-4 being intensified in the presence of IL-4δ2 manifested in that case agonist properties. The data obtained verified the existence of a system in the organism, which lessened the role of IL-4 full-sized form in polarization of immune response according to TH2-type.

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INTERLEUKIN-18 ATTENUATES THE ACUTE PANCREATITIS INDUCED BY CERULEIN IN MICE

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Recent studies indicated high correlation of circulating interleukin-18 (IL-18) with severity in human acute pancreatitis. However, the meaning of this phenomenon and the roles of IL-18 are not known. In this study, the effect of IL-18 on cerulein-induced acute pancreatitis in mice was examined. Administration of cerulein to wild type BALB/c mice caused histological changes in the pancreas and elevated serum levels of pancreatic enzymes, amylase and lipase and in the circulation of these mice high levels of IL-18 were also detected. In IL-18 deficient mice, cerulein caused severer pathological changes than in wild type BALB/c mice. Similarly, conditions of mice treated with neutralizing anti-IL-18 R antibody in advance were worse than those without antibody treatment, too. Administration of IL-18 to mice before or after injection of cerulein inhibited the increase of amylase and lipase as well as pancreatic histological changes. Levels of IL-10 in the pancreas of these mice were higher than those of mice without IL-18 injection. Thus, endogenous IL-18 was shown to regulate the inflammatory injury in the pancreas induced by cerulein, and exogenously given IL-18 attenuated the acute pancreatitis. IL-18 may regulate the inflammatory responses through induction of IL-10.

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IL-1F6, IL-1F8 AND IL-1F9 SIGNAL THROUGH IL-1Rrp2 TO ACTIVATE NFκB AND MAP KINASES

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Interleukin 1 plays a prominent role in immune and inflammatory reactions. The IL-1 family has recently been expanded to include 6 novel members named IL-1F5-IL-1F10. Recently, it was reported that IL-1F9 activated NFκB through the orphan receptor IL-1R-related protein (IL-1Rrp2) in Jurkat cells [1]. In this study, we demonstrate that IL-1F6 and IL-1F8, in addition to IL-1F9, activate NFκB in an IL-1Rrp2-dependent manner in Jurkat cells as well as in multiple other human and mouse cell lines. Activation of NFκB by IL-1F6 and IL-1F8 follows a similar time course as IL-1β, suggesting the activation of NFκB by these proteins occurs through a direct mechanism. Transfection of an IL-1RAcP dominant negative which blocked activation of NFκB by IL-1, also blocked activation by IL-1F6, F8 and F9. IL-1F6, F8 and F9 activate NFκB endogenously in a mammary epithelial cell line, NCI/ADR-RES cells, which express IL-1Rrp2 and IL-1RAcP. IL-1Rrp2 antibodies block the activation of NFκB by IL-1F6, F8 and F9 in both Jurkat and NCI/ADR-RES cells. In addition, IL-1F6, F8 and F9 activate the MAP kinases, JNK, p38 and ERK in NCI/ADR-RES cells. IL-1F6, F8 and F9 therefore activate NFκB and MAP kinases through IL-1Rrp2 and IL-1RAcP.

Reference

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IL-21 IN SYNERGY WITH IL-15 OR IL-18 ENHANCES IFN-γ PRODUCTION IN HUMAN NK AND T CELLS

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IL-21 is a T cell-derived cytokine the expression of which is regulated by T cell receptor stimulation. IL-21R shares the common cytokine receptor γ-chain (γ_c) with the receptors for IL-2, IL-4, IL-7, IL-9, and IL-15. Despite the same γ_c, these cytokines have different effects on diverse cells. We have previously shown that IL-15 and IL-21 induce the expression of IFN-γ, T-bet, IL-2Rα, IL-12Rβ2, IL-18R, and MyD88 genes in human NK and T cells. In addition, we and others have shown that IL-21 preferentially activates STAT3. NK and T cell-derived IFN-γ is a key cytokine that stimulates innate immune responses and directs adaptive T cell response towards Th1 type. We show here that in human NK and T cells IL-15 clearly activated IFN-γ mRNA expression and protein production. IL-18 and IL-21 enhanced IL-15-induced IFN-γ gene expression. IL-18 or IL-21 alone induced a modest expression of IFN-γ gene but a combination of IL-21 and IL-18 efficiently up-regulated IFN-γ production. IL-21 also induced the binding of STAT1, STAT3, and STAT4 to the regulatory sites of the IFN-γ gene. Our results suggest that both IL-15 and IL-21 have an important role in activating NK cell-associated innate immune response and Th1-associated T cell response.

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ACTIVITY REGULATION OF THE LYMPHOCYTE-ACTIVATED KILLERS OF IL-4 AND ITS ALTERNATIVE FORM IL-4δ2

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The influence of IL-4 and IL-4δ2 on the activity of LAK cells obtained from mononuclears of the peripheral blood at their incubation together with sub-optimal doses of IL-2 has been studied. Killer activity was tested according to their lytic effect on myelogenic leukemia cell line K-562, detecting the number of the viable cells evaluated by the level of formazan crystal formation from MTT. Maximal number of the active LAKs in control was reached on the 4-5-th day of mononuclear incubation. At the addition of 10 ng/ml of IL-4 into the culture medium, LAK activity was down-regulated as compared to the controls in 1.8-2.2 times. Addition of 10 ngγ/ml of IL-4δ2 to mononuclears incubated together with IL-2 did not produce any visible influence on the killer activity. IL-4δ2 concentration growth in the culture medium upto 100 ng/ml of increased the lytic index by 8-10% as compared to the controls and entirely canceled IL-4 suppressor effect on LAK activity.

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INTERLEUKIN-18 CONTROLS BLEOMYCIN – INDUCED LUNG INJURY

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Pulmonanry inflammation and following fibrosis caused by bleomycin is a difficult disorder whose pathophysiological mechanism remains obscure. It is also an important subject needed to be solved for prevention of side effects of chemotherapy in cancer. In this study, roles of interleukin-18 (IL-18) in pulmonary injury caused by bleomycin were investigated using wild-type and IL-18 deficient mice. We examined the severity of the pulmonary injury based on several parameters including myeloperoxidase activity, leukocyte population in the bronchoalveolar lavage and blood, histological images, and hydroxyproline contents in the lung. Bleomycin caused much severer lung injury in IL-18 deficient mice than in wild type mice, and the survival rate was much lower, and the body weight loss was also more marked in IL-18 deficient mice. The administration of IL-18 at various timing to the deficient mice treated with bleomycin ameliorated the severity of the pulmonary injury and improved the survival rate. Also in wild type mice, administered IL-18 in advance to bleomycin ameliorated the severity of pulmonary injury when scored by the above parameters. Administration of IL-18 at day5 post bleomycin instillation, inhibited the following fibrotic change. Pretreatment was the most effective on prevention of pulmonary inflammation. However, post treatment by IL-18 was still effective on prevention of pulmonary fibrosis. Thus, it was shown that IL-18 controls acute inflammatory reaction caused by bleomycin. Pretreatment of mice with IL-18 induced expression of IL-10 in the lung and spleen. These results indicate the regulatory roles of endogenous IL-18 in belomycin- induced pulmonary injury and that exogenously given IL-18 can ameliorate the severity of the inflammatory injury. The regulatory mechanism for inflammation in which IL-18 is involved will be discussed.

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THE STRUCTURE AND IMMUNOREGULATORY PROPERTIES OF HUMAN RECOMBINANT PROTHYMOSIN α

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The structural properties and conformational stability of recombinant human prothymosin α (ProTα) were characterized at neutral and acidic pH by gel filtration, SAXS, circular dichroism, ANS fluorescence, ^{1}H NMR, and resistance to urea-induced unfolding. ProTα underwent a cooperative transition from the unfolded state into a partially folded conformation on lowering the pH. That conformation of ProTα was a compact denatured state, with structural properties different from those of the molten globule. The formation of α-helical structure by the glutamic acid-rich elements of the protein accompanied by the partial hydrophobic collapse was expected at lower pH due to the neutralization of the negatively charged residues. Such conformational changes might be associated with the protein function.
ProTα prevented thymocyte apoptosis induced by dexametazone, stimulated T-cell maturation from their progenitors and manifested neuro-psychotropic characteristics on the systemic level, regulating the organism adaptation to stress impact.

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TEMPORAL ASSOCIATIONS BETWEEN IL-22 AND THE EXTRACELLULAR DOMAINS OF IL-22R AND IL-10R2

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IL-22 is a cytokine that has a putative role in inflammation. We studied by ELISA the interaction between IL-22 and the extracellular domains (ECD) of its receptor chains, IL-22R and IL-10R2. IL-22 has a measurable affinity for IL-22R. Cytokine binding to IL-10R2 alone was not detected. IL-22, however, has a stronger affinity for IL-22R/IL-10R2 ECD presented as Fc heterodimers. Further analyses suggest that IL-10R2 binds to a surface created by the association between IL-22 and IL-22R, further stabilizing the cytokine's interaction with its receptor. Neutralization of cytokine-receptor binding activity with either a rat IL-22 antibody or an Fc fusion to IL-22BP, the latter a secreted natural antagonist for IL-22, defines a cytokine epitope that may overlap the binding site for IL-22R on IL-22. Neutralization studies with a second rat IL-22 antibody define a separate IL-22 epitope that may overlap a binding site for IL-10R2. We propose a temporal model for the development of a functional IL-22 cytokine receptor complex.

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IL-4δ2 PARTICIPATION IN DENDRITIC CELL MATURATION REGULATION

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IL-4 plays an important pert in generation of dendritic cells (DC) from monocytes. IL-4δ2 is being a natural antagonist of IL-4. This work includes the study of IL-4δ2 regulatory role in DC maturation. Immature DCs were obtained from human peripheral blood monocytes using a standard technique in the presence of IL-4 (5 ng/ml) and GM-CSF 1000 MU/ml). Immature DCs were of round form and had $CD14^{low}CD83^{-}CD80^{-}CD86^{high}CD40^{+}$phenotype. Weak adhesion of the cells with plastic had been registered. Macrophages developed from monocytes under the influence of GM-CSF differed from immature DCs with angular form and more stable adhesion to plastic. They expressed the monocyte/macrophage CD14 marker stronger and did not carry CD40 on their surfaces. But significant amount of CD86 co-stimulator was detected on the macrophage surfaces, though less than on DCs. Initial monocytes also expressed CD86 but some order weaker than macrophages and immature DCs.

Further incubation of the immature DCs for 3-days under the influence of IL-4δ2 (5ng/ml) and GM-CSF (1000 MU/ml) resulted in the full loss of CD86 and CD40 on the DCs surfaces. However, no manifested changes in CD cell morphology were registered. CD14 molecule expression was on the previous level. The following incubation of the cells with a standard combination of IL-4 and GM-CSF for 3 days demonstrated that the content of co-stimulating molecules of CD86 and CD40 was fully restored on their surfaces.

Thus, IL-4δ2 as a natural antagonist of IL-4 reversibly suppressed expression of co-stimulating CD40 and CD86 molecules on the DCs surfaces.

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THE IL-1 HOMOLOGUES IL-1F7B AND IL-18 CONTAIN FUNCTIONAL MRNA INSTABILITY ELEMENTS WITHIN THE CODING REGION RESPONSIVE TO LPS

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We recently demonstrated that the interleukin-1 (IL-1) homologue IL-1F7b shares critical amino acid residues with IL-18 and binds to the IL-18 binding protein enhancing its ability to inhibit IL-18-induced interferon-γ. Here we show that similarly to IL-18 both mRNA and intracellular protein expression of IL-1F7b are upregulated by LPS and that 60-80% of both cytokines colocalize in human monocytes. We had evidence that in stable transfectants of murine RAW264.7 cells there was a 3'UTR independent control of IL-1F7b transcript stability. After LPS stimulation, there was a rapid transient increase in IL-1F7b-specific mRNA and concomitant protein levels. Using sequence alignment, we found a conserved 10 nucleotide homology box within the open reading frame of IL-F7b, which is flanking coding region instability elements of selective genes. In frame deletion of downstream exon 5 from the full length IL-1F7b cDNA markedly increased steady-state levels of IL-1F7b mRNA. A similar coding region element is located in IL-18. When transfected into RAW264.7 macrophages, IL-18 mRNA was also unstable unless treatment with LPS. These results indicate that both IL-1F7b and IL-18 mRNA contain functional instability determinants within their coding region, which influence mRNA decay as a novel mechanism to regulate the expression of IL-1 family members.

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HIGH-RESOLUTION STRUCTURE OF MURINE INTERLEUKIN 1 HOMOLOG IL-1F5 REVEALS UNIQUE LOOP CONFORMATION FOR RECEPTOR BINDING SPECIFICITY

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Interleukin – 1 (IL-1) F5 is a novel member of the IL-1 family. The IL-1 family are involved in innate immune responses to infection and injury. These cytokines bind to specific receptors and cause activation of NFκB and MAP kinase. IL-1F5 has a sequence identity of 44% to IL-1 receptor antagonist (IL-1Ra), a natural antagonist of the IL-1 system. Here we report the crystal structure of IL-1F5 to a resolution of 1.6Å. It has the same β-trefoil fold as other IL-1 family members and the hydrophobic core is well conserved. However, there are substantial differences in the loop conformations, structures that confer binding specificity for the cognate receptor to IL-1β and the antagonist IL-1Ra. Docking and superimposition of the IL-1F5 structure suggests that is unlikely to bind to the interleukin1 receptor, consistent with biochemical studies. The structure IL-1F5 lacks features that confer antagonist properties on IL-1Ra and we predict that like IL-1β it will act as an agonist. These studies give insights into how distinct receptor specificities can evolve within related cytokine families.

RECEPTORS

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ENHANCED EXPRESSION OF GRANULOCYTE INTERMEUKIN-10 RECEPTOR 1 DURING HUMAN ENDOTOXEMIA

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Background: IL-10 is an anti-inflammatory cytokine interacting with cells via a specific receptor composed of at least two subunits: IL-10R1 and IL-10R2. *In vitro* investigations showed that IL-10 responsiveness of cells is critically determined by IL-10R1, serving as a high-affinity binding receptor, whereas IL-10R2 mediates signal transduction only in the presence of IL-10R1.
Aim & Methods: To determine the effect of LPS on granulocyte IL-10R expression *in vivo*, 8 healthy male subjects received an iv. dose of LPS (4ng/kg). Blood was drawn for FACS analysis to measure granulocyte expression of IL-10R1, IL-10R2 and CD11b. Values as mean ± SE mean fluorescence intensity. Statistics by one-way ANOVA. *$p < 0.05$.
Results: LPS induced a granulocytopenia (1 h: $1.2 \pm 0.3 \times 10^9$/L) followed by a granulocytosis (8 h: $12.3 \pm 1.2 \times 10^9$/L*). LPS administration caused a increase in granulocyte IL-10R1 expression, peaking after 24 h (from 19 ± 6 to 94 ± 18*). Granulocyte IL-10R2 showed a non-significant decrease reaching a nadir after 9h (from 20 ± 4 to 12 ± 2). After LPS administration, granulocytes appeared in an activated state as reflected by an increase in surface CD11b expression (from 1000 ± 99 to 3500 ± 150 at 4 h*).
Conclusions: Human endotoxemia is associated with enhanced expression on granulocytes of IL-10R1, likely rendering them more sensitive to the anti-inflammatory effects of IL-10.

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MAP77/hOSCAR, A NOVEL FcRγ CHAIN ASSOCIATED RECEPTOR EXPRESSED BY MYELOID CELLS ACTIVATES EARLY IMMUNE RESPONSE

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Immune cell activity is regulated by both activatory and inhibitory immune receptors, which transduce signals through immunoreceptor tyrosine-based activating motifs (ITAM), and through immunoreceptor tyrosine-based inhibitory motifs, respectively. We have isolated a cDNA clone from an EST library of human monocyte-derived dendritic cells (m-DC) coding for a new member of the immunoglobulin superfamily. The molecule consists of an extracellular region containing two Ig-like loop domains, a short cytoplasmic domain, and a transmembrane domain with an arginine residue, similar to other activatory receptors associated with the FcRγ chain. The sequence has been registered as the human homologue of mouse osteoclast-associated receptor, OSCAR.
Expression studies by Northern blot, RT-PCR, and flow cytometer suggested restriction to myeloid cells, including DC. Immunoprecipitations with specific mAbs revealed a molecular weight of \\ 126\\40 kD. Co-immunoprecipitation experiments on m-DC showed association of MAP77/hOSCAR with the FcRγ chain, an ITAM-bearing adapter conferring an activatory potential.
Cross-linking of MAP77/hOSCAR on monocytes and m-DC induced Ca^{2+} flux and upregulation of maturation markers, as well as release of proinflammatory cytokines by monocytes. Moreover, m-DC internalised the receptor by endocytosis. All these features make MAP77/hOSCAR a new endocytic and activatory immunoreceptor, that could play a role in the early immune response.

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DEVELOPMENT OF A NOVEL BIOLOGICAL ASSAY FOR SWINE INTERLEUKIN-12

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Complementary and genomic DNAs encoding swine IL-12 receptor β2 (IL-12Rβ2) subunit were cloned, and the nucleotide sequences were determined. To confirm the biological function of encoded protein, the entire open reading frame (ORF) was re-cloned into a mammalian expression vector, and introduced to a Th1-like human lymphoma cell line, Jurkat E6-1. Antibiotic-resistant cells retaining the expression construct were selected then, isolated by the limiting dilution method. An established clone (10B10) constitutively expressed chimeric IL-12 receptors composed of intrinsic (human) β1 and extrinsic (swine) β2 subunits, and produced interferon (IFN)-γ in response to IL-12 of both species with optimal PMA/PHA stimulation. The production of IFN-γ was observed as early as 42 hours after culture and appeared to be dose-dependent within the range between 20-2000 pg/ml. Thus, this clone not only reacts with IL-12 of both species but also provides a useful tool for quick and sensitive detection of IL-12 bioactivity.
IL-12Rβ2 gene may be encoded by 15 exons (exon 2-16) with 5'-upstream untranslated exon (exon 1)
No TATA-box motif was observed within at least 1.5 kbp upstream of transcription start site (+ 1) in exon 1. Promoter activity of the upstream sequence has now been under investigation.

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ONCOSTATIN M REGULATES ONCOSTATIN M RECEPTOR EXPRESSION DURING ACUTE PERITONITIS

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Field or you can paste your text into the field. Text must not exceed 200 words.
The action of oncostatin M (OSM) is dependent upon the severity and duration of an inflammatory response. Recently, we have shown that OSM modulates leukocyte recruitment during peritonitis, which is not subject to regulation by soluble gp130, suggesting the involvement of other regulatory mechanisms. Using affinity chromatography, we have detected the presence of an OSM binding protein (\\ 126\\ 45KDa) in peritoneal fluid that was discovered to be a soluble isoform of OSM receptor (OSMR). Using an in-house ELISA we found a direct correlation between soluble OSMR and OSM expression in peritoneal fluid during the progression of peritoneal inflammation. Further experiments using FACs analysis, showed a differential temporal expression pattern of membrane-bound OSMR on peritoneal mesothelial cells. Plus RT-PCR analysis, using the same cells, revealed the induction (after 24hs) of not only membrane-bound OSMR transcript but also a spliced OSMR variant. Indeed, analysis of the supernatants revealed the presence of soluble OSMR. Since OSM is elevated during inflammation and known to regulate the expression of OSMR, these results suggest that OSM may regulate OSMR expression during peritonitis, which involves the expression of a putative soluble OSMR that may serve to modulate its action during an acute inflammatory response.

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SEMA6A, A NEW MOLECULE FOR ACTIVATED LANGERHANS CELLS

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Semaphorins represent a large family of molecules originally implicated in axon guidance. SEMA6A, a transmembrane molecule of class VI semaphorin which ligand remains unknown, displays signalling motif in its intracellular part and may act, contrarily to secreted semaphorins, as ligand or receptor.
We have performed RT-PCR analysis for different semaphorins in immune cells and have focused our attention on SEMA6A which appeared restricted to dendritic cells (DC) and up-regulated upon CD40L activation. To investigate SEMA6A expression and function, we generated monoclonal antibodies. SEMA6A expression was detected by flow cytometry on $CD34^+$-derived $CD1a^+$ DC subset and on TGF-β differentiated $CD14^+$ DC subsets, both closely related to skin Langerhans cells (LC). SEMA6A expression appeared on matured in *in vitro* generated LC and its expression was further enhanced after 48 hours of culture in presence of IFN-γ. IL-4 and IL-10, known to inhibit IFN-γ effect, reduced SEMA6A staining observed in presence of IFN-γ.
Thus, based on the role of semaphorins in axon guidance, this observation might suggest that SEMA6A plays a role in the imigration of the LC out of the skin or in the establishment of the network of maturing LC and interaction with T cells in lymphoid organs.

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THE SIGLEC FAMILY OF IMMUNE INHIBITORY RECPETORS

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Siglecs (sialic-acid binding immunoglobulin-like lectins) are immune inhibitory receptors expressed on the surface of cells of the haematopoietic system.. Each of the Siglecs characterised to date have an extracellular immunoglobulin (Ig) variable (V) domain and varying numbers of immunoglobulin constant (C2) domains. Their cytoplasmic tail contains one immunoreceptor tyrosine-based inhibitory motif (ITIM) and one signalling lymphocyte activation molecule-like motif (SLAM), suggesting that Siglecs possess signal transduction activity. The binding of SHP-1, SHP-2 and SHIP to the phosphorylated ITIM of CD33 (Siglec 3)-like Siglecs has been shown previously. These phosphatases bind via their SH2-domain. SOCS (suppressor of cytokine signalling) proteins are inhibitory proteins which contain a SOCS box motif and an SH2-domain homologous to the SH-2 domain of SHP-1 and SHP-2. It is our intention to show an association between the CD33-like Siglecs and the SOCS-3 protein using monoclonal antibodies developed against the Siglec family members and against SOCS-3. These antibodies will also be used to further characterise the Siglec family members, of which very little functional information is known.

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MECHANISM INVOLVED IN EOTAXIN-INDUCED INTERNALISATION AND RECYCLING OF THE CHEMOKINE RECEPTOR CCR3

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Chemotaxis mediated by chemokine receptor CCR3 plays a key role in eosinophil activation and migration into areas of inflammation in diseases such as asthma. Following ligand (eotaxin) binding to CCR3, cellular responses are rapidly attenuated. A variety of mechanisms may be responsible for signal attenuation including receptor desensitization, endocytosis and down-regulation. Recent evidence suggests that β-arrestin forms a complex with cSrc tyrosine kinases and that this is critical for chemokine receptor desensitisation and internalisation.
In this study we have delineated the CCR3 receptor localisation in intracellular compartments in RBL cells transfected with the human CCR3 receptor following stimulation with eotaxin. Immunofluorescence and FACS analysis demonstrate that in resting cells, most of the CCR3 is expressed on the plasma membrane, however, upon exposure to eotaxin the receptor is rapidly internalised and can be found in early endosomes. One hour after stimulation the receptor appears in a perinuclear structure while at 2 hours the receptor has recycled back to the plasma membrane. Studies using the Src-kinase inhibitors suggest that these kinases may be involved in the trafficking of the receptor from the endosome to the plasma membrane.

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DUAL FUNCTION AND SIGNALLING OF IL-17 IN SYNOVIAL FIBROBLASTS

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The memory T-cell derived cytokine IL-17 is described to share its profile of cellular functions with proinflammatory cytokines. Here we present evidence that IL-17 in addition also provides anti-inflammatory effects. The expression of TNF-induced VCAM-1, a molecule on the fibroblast surface enabling cell-to-cell contact with leukocytes, was significantly inhibited in the presence of IL-17. Functional neutralization of the cloned IL-17 receptor (IL-17R), by a monoclonal antibody, did not reverse the inhibitory effect of IL-17 on VCAM-1 expression. In contrast the synergistic effect of IL-17 on the TNF-induced IL-6 secretion and the down regulatory effect of IL-17 on TNF-induced Rantes secretion were competitively inhibited by the IL-17R blocking antibody. Ligand-binding studies revealed high affinity binding of IL-17 with a Kd value of 150pM (250 sites/cell), unlike the known low affinity receptor IL-17R. The novel receptor's signal transduction correlates with the protection of IkappaB beta degradation, but not of IkappaB alpha. We currently describe an anti-inflammatory function that is conveyed by a distinct fibroblast IL-17 receptor of high affinity associated with specific post-receptor signals. The novel synovial receptor provides a scientific basis for a selective intervention to counteract the detrimental effects of TNF in rheumatoid arthritis.

SIGNAL TRANSDUCTION

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CYTOREP: A UNIVERSAL BIOASSAY FOR THE IDENTIFICATION OF CYTOKINE RECEPTOR LIGANDS

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The clinical application of cytokines in the treatment of related pathologies revealed safe and therapeutically effective. Nevertheless, cytokines as therapeutic agents exhibit limiting characteristics such as difficult administration and strong side effects.
The identification of non-peptide molecules, able to mimic the natural ligand activity on the receptor and of muteins, with selective and sometimes novel biological properties, could give great advantages.
We have developed CytoRep: a convenient universal cytokine reporter bioassay for the identification of cytokine receptor modulators.
Cytokines mediate their responses through the JAK/STAT signalling pathway activation. The IL3 dependent pro-B murine BaF3 and the rat hepatoma H5 cell lines, which both express the majority of STAT proteins, have been used. A Luciferase reporter gene under the control of multiple copies of STAT3-STAT5 response elements has been permanently transfected in these cells. The reporter cell lines have been then transfected with different human cytokine receptors: G-CSF-, TPO-, EPO-, IL15- and IL21-receptors, showing up to 30 fold induction of Luciferase activity, upon 6 hours stimulation with the corresponding cytokines.
The two reporter cell lines have been optimized for both 96 and 384 wp format.
CytoRep represents an innovative, generic and convenient tool for high throughput identification of molecules capable of modulating different cytokine receptors.

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COMPARISON OF EPSTEIN-BARR VIRUS LATENT MEMBRANE PROTEIN COMPARISON OF LATENT MEMBRANE PROTEIN 1 AND TUMOUR NECROSIS FACTOR RECEPTOR 1 SIGNALLING

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The tumour necrosis factor receptors (TNFRs) are a superfamily of cell surface receptors, which have important roles in immune cell function. They lack any catalytic ability of their own, but recruit cytoplasmic adapter proteins through which the activation intracellular signalling pathways occur. Epstein-Barr virus (EBV) encodes a constitutively activated mimic of a TNFR, the latent membrane protein-1 (LMP1), which binds TNFR-associated adapter proteins. For the first time, we have compared LMP1 with the prototypic TNFR, TNFR1. Both LMP1 and TNFR1 activated NFκB complexes containing the same NFκB subunits, albeit with subtly different kinetics. A characterisation of the dominant negative properties of LMP1 and TNFR1 mutants revealed differences in the efficiency of a C-terminal truncation and point mutant of TNFR1, but no difference between analogous mutants of LMP1. An investigation of the expression and localisation of various TNFR1 and LMP1 constructs revealed that removal of the TNFR1 C-terminus greatly increased cell surface and overall expression. This was not observed with a similar mutant of LMP1. This data suggests that LMP1 and TNFR1 have different regulatory mechanisms, which probably reflect the nature of the receptor signalling. This is likely to have implications for the role played by LMP1 in malignancy.

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REGUALTION OF JAK ACTIVITY THROUGH UBIQUITINATON

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Haematopoietic cytokines signal through the activation of the *Janus kinase/Signal transducer and activator of transcription* JAK/STAT signal transduction pathway, and other signaling intermediates, resulting in gene induction and cellular activation. The *Janus Kinase* family consists of four members, JAK1, JAK2, JAK3 and TYK2 which are receptor bound and undergo phospho-tyrosine dependant activation following ligand stimulation. Modulation of JAK activity is essential in order to induce appropriate immune responses and to maintain homeostatic balance within the haematopoietic system. Several proteins have been shown to down-regulate JAK activity including members of the *Suppressors of Cytokine Signalling* (SOCS) family. Ubiquitination has emerged as a dynamic process which can modulate the activity, expression levels and sub-cellular localisation of proteins. SOCS1 and SOCS3 have been shown to regulate the ubiquitination of various substrates however, although they interact with the JAKs, JAK ubiquitination has not been reported. We demonstrate that phospho-tyrosine dependant JAK ubiquitination results in a reduction in JAK protein half-life. Furthermore, co-expression of SOCS3 strongly inhibits JAK activation and subsequently results in a reduction in the level of JAK ubiquitination. These findings suggest that ubiquitination may be an important mechanism by which both the activity and expression of these tyrosine kinases can be regulated.

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ROLES OF P21O7 PROTEIN ISOFORMS IN TRAF6-MEDIATED SIGNAL TRANSDUCTION

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TRAF6 is involved in the signalling mechanisms of the IL-1 receptor, the TLRs and certain cognates of the type I TNF receptor, such as CD40 or RANK. A key function of TRAF6 in these signalling pathways is to activate the TGFß-activated kinase 1 (TAK1). The kinase is recruited to TRAF6 by an adapter called TAK1-binding protein 2 (TAB2). We have recently mapped the TAB2-binding domain (TBD) of TAK1. A domain homologous to the TBD is present in two proteins encoded by the chr21orf7 gene, called p21o7-A and p21o7-D. This suggested that like TAK1, the two p21o7 proteins might associate with TRAF6 complexes via binding to TAB2. We have demonstrated binding of p21o7-D to TAB2 in both yeast and mammalian cells and shown that in the latter system, overexpression of p21o7-D inhibits activation of TAK1 by IL-1, TNF and LPS stimulation, or by co-transfected TRAF6. More recently, we have also cloned the other TAB2-binding isoform, p21o7-A. It contains an amino-terminal domain that is absent from p21o7-D, suggestive of an active signalling role for p21o7-A in TRAF6 signalling. The function of p21o7-A is currently being investigated.

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INTERLEUKIN-3, COLONY STIMULATING FACTOR – 1 AND EPIDERMAL GROWTH FACTOR PREFERENTIALLY ACTIVATE M-RAS AND K-RAS 4B OVER H-RAS

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The four isoforms of p21 Ras, H-Ras, N-Ras and K-Ras 4A and 4B, together with other members of the Ras sub-family such as M-Ras, share positive and negative regulators as well as effector molecules. There is little information on which of the isoforms of p21 Ras or other members of the Ras sub-family are activated by growth factors, as most studies of Ras activation used an assay that failed to discriminate between p21 Ras and other members of the sub-family. We observed that interleukin-3, colony-stimulating factor – 1 and epidermal growth factor all preferentially activated M-Ras and K-Ras 4B which have polybasic carboxy termini over H-Ras (which is palmitoylated). We used chimeric Ras proteins in which we had switched carboxy termini between M-Ras and H-Ras and K-Ras 4B and H-Ras to show that the presence of a polybasic carboxy terminal tail was sufficient to favor activation by growth factors and to dictate localization in the disordered regions of the membrane rather than in lipid rafts. We noted that the receptors for these growth factors all tended to be localized in disordered membranes, suggesting that co-localization of receptors and those Ras isoforms with polybasic carboxy termini in disordered membranes might favor activation of these isoforms by growth factors. These results suggest that the differential localization of different Ras isoforms in sub-regions of the membrane may influence their activation by ligands.

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THE ROLE OF THE GAB-2 PH DOMAIN IN IL-3 SIGNALLING

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Gab-2 plays a critical role in the recruitment of class I_A phosphoinositide 3-kinases (PI3Ks) to the IL-3 receptor. However, the mechanisms by which Gab-2 is recruited and the role of its PH domain are still unclear. The PH domain of Gab-1 can bind $PI(3,4,5)P_3$, localising it to the plasma membrane where it is phosphorylated and associates with receptor complexes. However, despite a highly homologous PH domain in Gab-2 its binding specificity to membrane phospholipids has not been determined.

The role of the Gab-2 PH domain in membrane localisation, IL-3-induced Gab-2 tyrosine phosphorylation, proliferation and chemotaxis has been investigated. Myc and GFP-tagged wild type Gab-2 or Gab-2 lacking the PH domain (ΔPH) have been inducibly expressed in IL-3-dependent BaF/3 cells. Deletion of the PH domain had little effect on IL-3-induced Gab-2 tyrosine phosphorylation, but led to increased association with SHP-2 and PI3Ks. MAPK, PKB activation and overall proliferation were unaffected. In membrane localisation studies both WT and ΔPH Gab-2 were detected at the membrane in resting cells but rapidly disappeared following stimulation. Preliminary studies show that deletion of the PH domain alters SDF-1 and IL-3-induced chemotaxis. Studies to assess the phospholipid binding specificity of Gab-2 PH domain are in progress.

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CHEMOKINES REGULATE INNATE IMMUNITY THROUGH SUPPRESSOR OF CYTOKINE SIGNALLING-1 (SOCS-1)

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Suppressors Of Cytokine Signalling (SOCS) are encoded by immediate early genes that are known to inhibit cytokine responses. These proteins are induced by a large number of cytokines and subsequently inhibit signalling, as part of a feedback loop. Although SOCS gene expression has been shown to be induced by a number of cytokines, growth factors and innate immune stimuli such as lipopolysaccharide (LPS), whether their expression is induced by chemotactic stimuli has not been reported.

In this study we report that the chemoattractants Interleukin-8 (IL-8) and N-formyl-Methionyl-Leucyl-Phenylalanine (FMLP) upregulate SOCS-1 mRNA and protein in myeloid cells and human neutrophils. These chemotactic factors did not induce the expression of any other SOCS besides SOCS-1.

As chemoattractant induced SOCS-1 expression in neutrophils may play an important role in regulating the subsequent response to bacterial products such as LPS and growth promoting cytokines like Granulocyte-Colony Stimulating Factor (G-CSF) we investigated the effect of chemoattractant-induced SOCS-1 on their signal transduction. We show that SOCS-1 suppressed both the LPS and G-CSF signal transduction pathways. This provides evidence for a cross-talk between the chemoattractant signal transduction pathway and both the Toll-Like Receptor-4 (TLR4) and cytokine signal transduction pathways involving SOCS proteins.

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FLAGELLIN IS A MAJOR DETERMINANT IN ACTIVATION OF THE PROINFLAMMATORY SIGNALING CASCADE AND GENE PROGRAM

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Infection of intestinal epithelial cells (IECs) by pathogenic *Salmonella* leads to potent activation of signaling cascades and the transcription factor NF-κB that initiate the proinflammatory gene program (PGP). *Salmonella* activates NF-κB during infection of cultured IECs and we found that flagellin produced by the bacteria leads to NF-κB activation in all the cells; bacterial invasion is not required. Flagellin alone activated the MAPK, SAPK and IKappa B kinase signaling pathways that lead to expression of the PGP in a temporal fashion nearly identical to that of infection of IECs by *Salmonella*. Flagellin expression was required for *Salmonella* invasion of IECs and it activated NF-κB via toll-like receptor 5 (TLR5). A number of cell lines that we found to be unresponsive to flagellin express TLR5 and expression of exogenous TLR5 in these cells induces NF-κB activity poorly in response to flagellin challenge. Conversely, overexpression of dominant-negative TLR5 only partially blocks NF-κB activation by flagellin. These observations are consistent with either a very stable TLR5 signaling complex or the existence of a limiting flagellin co-receptor or required adapter or both. These collective results provide evidence that flagellin acts as a main determinant of *Salmonella* mediated NF-κB and proinflammatory signaling and gene activation.

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PHOSPHORYLATION SITE-SPECIFIC IMMUNOASSAY FOR STUDYING STAT1 ACTIVATION AND CORRELATION TO CYTOKINE EXPRESSION

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STATs (signal transducer and activator of transcription) are a family of transcription factors that have diverse roles in numerous cellular processes including immune response, cell growth, differentiation, cell survival, apoptosis, and oncogenesis. To facilitate research in these areas of study, we developed a Stat1[pY701] ELISA to detect and quantify the cellular level of phosphorylated Stat1 as an alternative to Western blotting. This assay is constructed using a pan-Stat1 specific monoclonal antibody and a phosphorylation site-specific antibody to Stat1[pY701]. Specificity of the Stat1[pY701] assay for phosphorylated Stat1 is confirmed by peptide competition. Using this assay, HeLa cells were treated with increasing doses of IFN-γ, and evaluated. Increased levels of pY701 were measured in a dose dependent manner, but not in untreated cells. Treatment of cell cultures with the tyrosine phosphatase inhibitor, sodium vanadate, prevents Stat1 dephosphorylation and results in elevated levels of Stat1[pY701]. This immunoassay detects phosphorylated Stat1 at a level 4 times more sensitive than Western blot. Since Stat1 phosphorylation and cytokine expression can impact each other, human PBMCs were treated with specific mitogens and the expression pattern for nearly 30 cytokines was analyzed over a time-course using BioSource Multiplex Bead Immunoassays. The correlation between Stat1[pY701] and cytokine expression will be investigated.

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DIFFERENTIAL EFFECT OF KAPPA SITES ON STIMULUS INDUCED TNFa EXPRESSION IN PRIMARY HUMAN MACROPHAGES

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TNFα has been well defined as a potent pro-inflammatory cytokine. It confers protection from microbial infection but is also involved in the pathogenesis of inflammatory disease such as Rheumatoid Arthritis, Crohns disease. To achieve this functional dichotomy between infection and inflammation, transcription and translation must be tightly regulated. It is well established that NF-kappaB plays a central role in the transcriptional regulation of TNFα. The human TNFα promoter region contains five sites, which have been defined as NF-kappaB binding sites (kB1, kB2, ξ, kB2a and kB3). We generated luciferase reporter constructs, linked to the full-length human TNFα promoter, containing the 3'UTR. Using an adenoviral delivery system in primary human M-CSF treated monocytes, it was shown that these sites did not contribute equally to the regulation of TNFα expression. Point mutations in each of the kappa sites, which abolished NF-kappaB/DNA binding, demonstrated that kappa sites kB2, kB2a and kB3 had the most profound affect on LPS induced TNFα luciferase activity in M-CSF treated monocytes. Other pro-inflammatory stimuli such as PMA and IL-1 were also investigated. Our studies highlight the possibility of decreasing TNFα production through specifically targeting areas of the TNFα promoter.

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HUMAN DUB DEUBIQUITINATING ENZYMES ARE GROWTH FACTOR REGULATED

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The DUB family of deubiquitinating enzymes were originally identified as murine growth factor inducible immediate early genes. DUB-1 is induced by IL-3, IL-5 and GM-CSF and expressed in a range of haematopoietic cell types, whilst DUB-2 is induced in response to IL-2 and appears to be expressed only in T-cells.

Previously we have reported that DUB-2 is expressed in HTLV-1 transformed T-cells that show constitutive activation of the IL-2 JAK/STAT pathway and that its expression in Ba/F3 cells markedly prolongs IL-2 induced STAT5 phosphorylation and inhibits apoptosis induced by cytokine withdrawal.

Recently we have identified a number of human ORFs which appear to be members of this family of proteins and confirmed their expression at the mRNA level. Here we demonstrate that these ORFs are expressed at the protein level and that their expression is inducible at both the mRNA and protein level in response to growth factor stimulation. In addition we also show that their expression in Ba/F3 cells blocks cell growth and increases the rate of apoptosis.

These observations suggest that the human members of the DUB family of deubiquitinating enzymes are growth factor inducible immediate early genes which influence both cell proliferation and survival.

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DIFFERENTIAL POST-TRANSCRIPTIONAL REGULATION OF TNF-alpha AND COX-2 GENE EXPRESSION IN RAW 264.7 CELLS

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Gene expression can be subject to multiple mechanisms of post-transcriptional control such as hnRNA splicing, nucleocytoplasmic transport, mRNA stabilization and translation. We have shown that, in the mouse macrophage-like cell line RAW 264.7, Tumour necrosis factor α (TNFα) mRNA stability is regulated by LPS-induced p38 MAPK activation and this regulation is mediated via an AU-rich element (ARE) in the 3'-untranslated region (3'UTR). We have also shown that cyclooxygenase-2 (Cox-2) mRNA stability is p38 MAPK regulated via a 3'UTR ARE in HeLa cells. We now show that, although Cox-2 protein expression is p38 MAPK-regulated, Cox-2 mRNA stabilization is not p38 MAPK-dependent in RAW 264.7 cells.

We have shown that TNFα and Cox-2 mRNA 3'UTRs bind similar ARE-binding proteins (ARE-BPs) (e.g. Tristetraprolin, HuR, AUF-1/AUF-2, hnRNPA1) using cell lysates and exogenous riboprobes in electrophoretic mobility-shift assays. However, the *in vivo* interactions of full-length TNFα and Cox-2 mRNAs and ARE-BPs are poorly understood.

To understand why the two ARE-containing mRNAs are differentially regulated we have undertaken a systematic analysis of the *in vivo* interactions of TNFα Cox-2 and GAPDH (control) mRNAs and ARE-BPs in RAW 264.7 cells using a panel of anti-ARE-BP antibodies, RNA/protein-complex immunoprecipitation and RT-PCR. Specific, regulated mRNA/protein interactions will be discussed.

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IDENTIFICATION OF CYTOKINE SIGNALING PATHWAYS WHICH MODULATE B CELL RESPONSES IN NORMAL AND LEUKEMIC B CELL LINES USING BIOSOURCE MULTIPLEX BEAD IMMUNOASSAYS

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B cell receptor (BCR) engagement by foreign antigen stimulates several cellular responses including enhanced proliferation, differentiation, and apoptosis. BCR engagement produces phosphorylation of numerous sites of the scaffolding protein BLNK, creating docking sites for the enzymes PLCγ, Vav, and Btk, and the adaptor proteins Nck and Grb2. The importance of BLNK is underscored by the observations that its deletion attenuates the Ca^{2+} flux and MAPK activation observed with BCR engagement. Enzyme activities which modify BLNK phosphorylation include Syk and SHP-1. Differential BLNK phosphorylation in response to BCR engagement and its modulation by the T cell-derived cytokines IL-6, interferon-gamma, and CD40L in normal and leukemic human B cell lines were observed by Western blotting using phosphorylation site specific antibodies. Further, we delineated signaling pathways leading to modulation of BLNK phosphorylation using the signal transduction inhibitors PP1 (Src family inhibitor), wortmannin (PI3 kinase inhibitor), LY294002 (PI 3 kinase inhibitor), Gö6976 (conventional PKC inhibitor), and U0126 (ERK1/2 inhibitor). Changes in downstream signaling through p38, JNK, and AKT, as well as cytokine production profiles in response to stimuli and inhibitors were assessed using Multiplex Bead Immunoassays. Differences between normal and leukemic cell lines and the biological implications will be discussed.

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GENERATION OF RAPAMYCIN RESISTANT T-CELLS

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The cytokine Interleukin-2 (IL-2) plays an essential role in the proliferation of cytotoxic T-cells (CTLs). The immunosuppressive drug rapamycin inhibits IL-2 signalling through inhibition of mammalian target of rapamycin (mTOR). However, the use of immunosuppression can result in complications associated with increased viral disease due to the lack of CTLs. Post-transplant, the lack of Epstein-Barr virus specific CTLs (EBV-CTLs) can result in Post-Transplant Lymphoproliferative Disease (PTLD).
It is our aim to develop a strategy to promote the function and longevity of CTLs by the introduction of a rapamycin resistant TOR (RR TOR) molecule using a retroviral system.
We have retrovirally infected primary human T-cells, EBV-specific CTLs and Kit 225 cells; an IL-2 dependent T-cell line, with a GFP retrovirus. Using a retroviral vector carrying the RR TOR gene we have generated a T-cell line expressing the RR TOR protein. Preliminary data suggests that the RR TOR gene is conferring a degree of rapamycin resistance on the T-cells. The next phase is the generation of EBV-CTL expressing RR TOR. This strategy may allow the generation of immunosuppression resistant CTL, which will function more efficiently in the T-cell immunotherapy of PTLD in the presence of immunosuppression.

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REGULATION OF EXPRESSION OF SUPPRESSORS OF CYTOKINE SIGNALLING (SOCS) 4-6 IN HAEMATOPOIETIC CELLS.

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Chronic inflammation involves the breakdown of normal regulation of inflammatory cells such as neutrophils, macrophages, dendritic cells, and T and B lymphocytes. A family of regulatory proteins termed suppressors of cytokine signalling (SOCS) is thought to be important for regulating the activity of these immune cells. The SOCS family consists of eight proteins, SOCS1-7, and cytokine inducible Src homology domain 2 (SH2)-protein (CIS), which contain a variable amino terminal, a SH2 domain, and a novel conserved carboxyl terminal motif called the SOCS box. It has been well documented that the expression of CIS, SOCS1 and SOCS3 are induced in response to stimulation by a wide variety of cytokines, and overexpression of these proteins in cells lines results in inhibition of cytokine signalling. To date, SOCS4-7 remain poorly understood and little is known about their function or mechanism of action.
We used different stimuli such as endotoxin, chemotactic factors and cytokines to determine whether SOCS4-6 are also regulated. Quantitative RT-PCR was used to determine mRNA expression and Western Blot analysis was used to determine protein expression levels of SOCS4-6. Our data suggests that in many cell types, including PMNs and PBMCs, these SOCS are constitutively expressed. The regulation of these SOCS by cytokines and growth factors suggest that they may also have an essential role in controlling the immune response.

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REGULATION OF TRANSCRIPTION FACTORS IN HUMAN HERPESVIRUS (HHV)-6B-INFECTED T CELLS

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Infection with human herpesvirus (HHV) 6B involves complex virus-host interactions visualized by the appearance of slightly enlarged cells and large syncytia. During the viral life cycle the viral genes are transcribed in a sequential manner with several of the viral gene products having transactivating potential. Like in infections with other members of the human herpesvirus family, several cellular functions are perturbed, but the molecular mechanisms during HHV-6B-infection are largely unknown. We have observed a block in the cell cycle of HHV-6B-infected T cells, which do not proceed into the S phase of the cell cycle. Others have shown that HHV-6B up-regulates several cellular genes characteristic for a pro-inflammatory response.
Among the more well-described members of the human herpesvirus family, it is now known that infection with herpes simplex virus, Epstein-Barr virus, and Human cytomegalovirus up-regulates the transcription factor NF-kappaB. Likewise, Varicella-zoster virus strongly activates the AP-1 components *Jun* and *Fos*.
Here we investigate the signaling pathways in HHV-6B-infected T cells. Results from the initial studies including characterzation of the DNA-binding activity of the transcription factors AP-1, NF-AT, and NF-κB by an ELISA-based method will be presented.

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}INHIBITION OF IL-1 INDUCED GENE EXPRESSION BY SUPPRESSOR OF CYTOKINE SIGNALING-3

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Interleukin-1 (IL-1) is believed to be involved in beta-cells destruction in type 1 diabetes by inducing pro-apoptotic genes. Suppressors of cytokine signaling (SOCS) are able to inhibit JAK/STAT signaling, but SOCS-3 has also recently been shown to inhibit IL-1 signaling in pancreatic beta-cells. In order to analyze the inhibitory effects of SOCS-3 on IL-1 signaling we performed microarray analysis of cells exposed to IL-1 in the absence or presence of SOCS-3. Stimulation by IL-1 resulted in the change in gene expression (> 2-fold) of more than 100 genes including iNOS, MnSOD, IRF-1, GADD153, c-myc, IκBa and p105. Of these genes the expression of iNOS, IRF-1, GADD 153, p105 and c-myc was reduced significantly (\\ 126\\50%) by SOCS-3, whereas the expression of MnSOD and IκBa was unaffected. This pattern of IL-1 and SOCS-3 regulation of gene expression was verified by quantitative PCR. Western blot analysis revealed that the expression of iNOS protein was induced by IL-1 and this up-regulation was inhibited by SOCS-3. IL-1 regulates transcription by induction of the transcription factor NFκB and gel-shift analysis showed that SOCS-3 was able to inhibit IL-1 induced activation of NFκB. In conclusion, SOCS-3 is able to suppress a subset of IL-1 regulated genes possibly by inhibiting the induction of NFκB.

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CREB1 LINKS INTERLEUKIN-2 AND PHOSPHATIDYLINOSITOL 3-KINASE TO CYCLIN D2

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Interleukin-2 regulates lymphocyte proliferation through phosphatidylinositol 3-kinase (PI3K). Using human peripheral blood lymphocytes and an IL-2 dependent T-cell lines, Kit225, we have identified a molecular mechanism by which PI3K links interleukin-2 to the synthesis of D-type cyclins, the first set of cell cycle proteins induced in T-lymphocyte proliferation. LY294002, a PI3K inhibitor, prevents the induction of cyclin D2 and cyclin D3 protein and mRNA. An active molecule of PI3K is able to transactivate the *cyclin D2* promoter at a site that binds CREB-1. IL-2 and PI3K regulate CREB phosphorylation which correlates with transcriptional activity. A dominant negative CREB prevents IL-2 induction of the cyclin D2 romter. Together this data suggests that CREB is a key link between IL-2, PI3K and cyclin D2 and thus regulates cell proliferation.

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CYBR, A CYTOKINE INDUCIBLE PROTEIN REGULATES DENDRITIC CELLL DEVELOPMENT AND T CELL PROLIFERATION

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Cytokines regulate the development and differentiation of cells of the immune system, but precisely how they control these processes is still poorly understood. Using microarray technology, we identified Cybr, a cytokine-inducible gene expressed exclusively in hematopoietic cells and tissues. Cybr is expressed in resting T, B, monocytes, macrophages, and dendritic cells, and its expression is markedly upregulated by cytokine stimulation or Toll-like receptor ligation. In the thymus, Cybr is preferentially expressed in single-positive thymocytes. Furthermore, Cybr is preferentially expressed in T helper 1 (Th1) cells. Cybr encodes two protein interaction domains: a PDZ domain and a coiled-coil domain. We have previously shown that Cybr interacts with cytohesin-1, a GTP-exchange protein for the ARF family of small GTPases, through interaction between the coiled-coil domains of the two proteins. Because of the known role of cytohesin-1 in LFA-1-mediated adhesion, Cybr was believed to also play a role in adhesion. Characterization of Cybr-deficient mice shows no alteration in cellular adhesion. Instead, Cybr knock-out mice show an increased TCR-induced T cell proliferation. We also observed a dendritic cells developmental defect and a impaired response to LPS. These data indicate that Cybr plays a critical role in the development and function of cells of the immune system.

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SOCS2 AN INHIBITOR OR NOT?

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Cytokines exert their biological effects by binding to specific cell surface receptors and activating the JAK/STAT signal transduction pathway. In order to appropriately control cellular responses it is important for a cell to terminate these signals. The recently identified family of proteins termed suppressors of cytokine signaling (SOCS) are upregulated by and inhibit the JAK/STAT pathway in a classical negative feedback manner.
SOCS3 has been shown to inhibit signalling by binding to the receptor and the activated Jak kinase. The mechanism of action of SOCS2 is not known, however it has been shown to be expressed in T cells and that this expression is induced by IL-2, -4, -5, -10 and GH. Here we demonstrate that SOCS2 and SOCS3 are induced under similar conditions but with different kinetics, with SOCS3 being induced much earlier than SOCS2 following stimulation. Furthermore we show that unlike other SOCS family members SOCS2 expression enhances STAT phosphorylation and therefore activation in cells following cytokine treatment. Expression of SOCS2 results in a reduction of SOCS3 protein expression in response to cytokines while SOCS3 mRNA is unaffected. It is therefore reasonable to postulate that SOCS2 may regulate SOCS3 protein expression in a proteasomal dependent manner and SOCS2 appears to potentiate signalling.

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PRE-ASSOCIATION OF NON-ACTIVATED STAT3 MOLECULES DEMONSTRATED IN LIVING CELLS USING BRET: A NEW MODEL OF STAT ACTIVATION?

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Signal transducers and activators of transcription (STAT) are crucial molecules in cytokine signaling. The conventional model of STAT activation says that the STAT molecules are recruited from a latent pool of cytoplasmic monomers to the activated cytokine receptor. After binding to the receptor they get tyrosine phosphorylated, dissociate from the receptor and translocate to the nucleus as activation-induced dimers. Recently, several publications questioned this model of STAT activation and showed the existence of pre-associated STAT molecules prior to activation. We were able to demonstrate the existence of pre-associated STAT3 molecules in living mammalian cells using Bioluminescence Resonance Energy Transfer (BRET). Our results support the new hypothesis that STAT molecules exist in the cytoplasm as dimers or multimers and point to an activation-induced change in STAT3 conformation. Therefore, we propose a new model of STAT activation and discuss a hypothetical structure of « cytoplasmic » STAT dimers as opposed to the known « activation-induced » dimer.

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BONE MARROW FIBROBLASTS (BMF) SUPPORT SURVIVAL OF B-CLL CELLS THROUGH ACTIVATION OF PI3-KINASE PATHWAY

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In spite of the long life span of B-CLL cells and resistance to apoptosis *in vivo*, the cells die rapidly in culture. Here, we describe an *in vitro* culture system which support ; urvival of B-CLL cells. B-CLL cells were co-cultured with BMF under serum free conditions for variable durations ranging from 16 hrs to 4 months. Flowcytometric analysis were used to assess cell viability (annexin V/propidium iodide). Co-cultivation of B-CLL cells with BMF increased significantly cell viability in comparison to suspension cultures ($p < 0.01$). Experiments using transforming growth factor-beta ,TGF beta (1-5 ng/ml) or neutralizing anti-TGF-beta antibodies showed that this cytokine inhibits apoptosis of B-CLL cells in presence of BMF but not in suspension cultures, pointing to an indirect protective role for TGF-beta. Since phosphatidylinositol 3-kinase (PI3-K) pathway is known to exert an antiapoptotic function upon ligation of integrins, cytokines and growth factor receptors, the leukemic cells were treated in co-cultures with PI3-K inhibitors; wortmannin or Ly294002. This led to inhibition of the supportive function of the fibroblasts and induction of apoptosis in B-CLL cells. The results demonstrate that human BMF support B-CLL cell survival and mimic the situation in the lymphoid tissues. The results also suggest that PI3-K is involved in the protection of B-CLL cells from apoptosis.

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NCAM/FGFR SIGNALING FACILIATES IL-1β MEDIATED MAPK ACTIVATION IN THE β-CELL LINE INS-1E

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Background/aims: Exposure of β-cells to cytokines activates MAPKs and causes apoptosis. In neurons, activated NCAM cis-activates the FGFR, leading to activation of ERK, an increase in intracellular Ca^{2+}-concentration and anti-apoptosis. Since β-cells express NCAM, we investigated if specific inhibitors of FGFR1, SU5402 (SU) or of NCAM mediated Fyn signaling, PP2, as well as the NCAM agonist C3d affected IL-1β induced MAPK in β-cells.
Methods: Rat insulinoma INS-1E cells were exposed to rmIL-1β ± SU/PP2/C3d. The MAPKs JNK and ERK were investigated by an *in vitro* phosphotransferase assay and verified by Western Blotting with phosphospecific antibodies.
Results: Pretreatment of INS1E-cells with SU (24 uM), but not PP2 (1 uM), inhibited IL-1β (160 pg/ml) mediated MAPK activation. Surprisingly, soluble (10 uM) or matrix bound C3d did not activate ERK, but inhibited IL-1β induced MAPK activation, suggesting that C3d in β-cells acts as an antagonist probably by breaking NCAM homodimerisation and activation.
Conclusion: NCAM/FGFR signaling facilitates IL-1β mediated MAPK activation, and the C3d peptide may have a therapeutic potential as inhibitor of cytokine-induced proapoptotic signaling.

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DISPARATE EFFECTS OF PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR γ LIGANDS ON PI3K AND MAPK SIGNALLING PATHWAYS

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Peroxisome-proliferator activated receptors (PPARs) are ligand dependent transcription factors with potential antiinflammatory actions. This study examines the activation of mitogen-activated protein kinase (MAPK) and phospatidylinositol-3 kinase (PI3K) signalling pathways by PPARγ ligands. We report that A549 (human lung epithelial cell line) express PPARγ and isoforms of PI3K (p85, C2α and C2β). *In vitro* lipid kinase assays show that both 15-deoxy-$\Delta^{12,14}$prostaglandin J_2 (15d-PGJ_2) and troglitazone can activate PI3K-C2α, and troglitazone can also activate PI3K-C2β. Further studies are required to investigate class 1A activity. 15dPGJ_2 and troglitazone at 10 iM enhanced the phosphorylation of PKB (a PI3K dependent downstream effector). Troglitazone, unlike 15d-PGJ_2 enhanced phosphorylation of ERK above basal levels and markedly stimulated cyclooxygenase-2 (COX-2) expression. 15d-PGJ_2 had no effect on COX-2 expression alone but inhibited TNFα induced COX-2 expression by approximately 50%, possibly by its known inhibitory action on NFκB. The disparate effects of the two ligands on COX-2 expression may suggest they are acting by different mechanisms, involving PPARγ independent effects.

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INTERLEUKIN-1 RECEPTOR-ASSOCIATED KINASE-1 (IRAK-1): AN ADAPTER PROTEIN THAT REGULATES ITS OWN AVAILABILITY THROUGH SEQUENTIAL PHOSPHORYLATIONS

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The interleukin-1 receptor-associated kinase 1 (IRAK-1) is a central adapter in the signaling complex of the Toll/interleukin-1 receptor family. Formation of this complex results in the activation and phosphorylation of IRAK-1. However it has been demonstrated that the enzymatic activity of IRAK-1 is dispensable for IL-1 signaling, leaving the role of its kinase activity as ill defined as the sites of IRAK-1 phosphorylation. We describe the molecular dissection of two properties of IRAK-1. We demonstrate that an IRAK-1 mutant containing only the death domain and the C-terminus suffices to activate NF-κB and to induce cytokine synthesis. We define sequential phosphorylation steps in IRAK-1, which are at least in part autophosphorylation. Initially, IRAK-1 is phosphorylated at T209, a site critical for its kinase activity, which results in a conformational change allowing subsequent phosphorylation of T387 in the activation loop leading to full kinase activity. Finally, IRAK-1 autophosphorylates several times in the ProST region. Hyper-phosphorylation of IRAK-1 leads to dissociation from the adapter MyD88 and the silencer Tollip, as well as to initiation of proteolytic cleavage. This identifies IRAK-1 as a novel type of adapter protein, which employs its own kinase activity to regulate its availability through sequential autophosphorylation.

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AGE-RELATED IMPAIRMENT IN LONG-TERM POTENTIATION MAY BE EXACERBATED BY TREATMENT WITH BETA-AMYLOID IN RAT HIPPOCAMPUS

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Evidence indicates that beta-amyloid (Aβ), the main constituent of neuritic plaques found in Alzheimer's Disease patients, may be responsible for the neuronal loss apparent in this disease. The effects of Aβ on cultured cells and on signaling events *in vivo* have been shown to depend at least in part on activation of the proinflammatory cytokine interleukin-1β (IL-1β) and subsequent activation of the stress-activated protein kinase c-jun N-terminal kinase (JNK). We have investigated the effects of Aβ(1-40) on signaling events in hippocampus and on long-term potentiation (LTP) in perforant path-granule cell synapses of the dentate gyrus in young, middle-aged and aged rats *in vivo*. Our data show that intracerebroventricular injection of Aβ (0.1nmol) inhibits LTP in middle-aged and aged rats but not young rats. The evidence indicates that hippocampal IL-1β concentration and JNK activity are increased with age and Aβ treatment and that these increases are negatively correlated with the ability of middle-aged and aged rats to sustain LTP. We propose that the effects of the low dose Aβ on LTP in middle-aged and aged rats is a consequence of the synergy between Aβ and increased endogenous IL-1β in the ageing brain.

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INTRACELLULAR SIGNALING INDUCED BY HERPES SIMPLEX VIRUS INFECTION: INDUCTION OF CYTOKINE AND CHEMOKINE PRODUCTION

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Previously, we have found a selective induction of the CC chemokine RANTES/CCL5 in macrophages after herpes simplex virus (HSV) infection, and that this induction was dependent on viral ICP0 and cellular double-stranded RNA-activated protein kinase (PKR) (J.Virol. 76: 2780). Further studies have shown the induction of CCL5 in fibroblasts and macrophages to be at the transcriptional level, and to be dependent on the transcription factors IRF-3 and NF-kappaB (J.Gen-.Virol, *In press*).
Here, we present further data on the signaling pathways activated upon HSV infection. Signaling pathways, which ultimately result in chemokine transcription and secretion – and initiation of an antiviral response.

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A 16-RESIDUE MIF-DERIVED PEPTIDE WITH MIF-LIKE BIOLOGICAL ACTIVITIES

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The mechanism of action of the inflammatory cytokine MIF is incompletely understood. Recently, JAB1/CSN5 and CD74 were identified as intracellular and membrane-bound MIF binding proteins, respectively, and JAB1- and CD74-mediated pathways were found to participate in MIF signal transduction processes. However, the molecular details of these mechanisms as well as potential contributions of MIF's enzymatic activity have largely remained unclear. We previously observed that a 16-meric MIF-derived peptide, MIF(50-65), competed with MIF for JAB1 binding. This finding prompted us to perform a structure activity analysis of this peptide and investigate the hypothesis that MIF(50-65) could more generally exhibit MIF activities. MIF(50-65), which spans the CXXC motif of MIF, reduced insulin through CXXC-dependent catalyis. Consequently, MIF(50-65) exhibited a negative redox potential (– 0.258 V). MIF(50-65) formed a disulfide-mediated beta-turn that was critical for most MIF-like activities analyzed. The peptide not only had catalytic redox activity and bound to JAB1, but intriguingly, was also found to activate glucocorticoid overriding and cell proliferation, enhance MAPK activation and stabilize p27Kip1 levels. Thus, MIF(50-65) is a surprisingly small MIF peptide that can mimick several important target cell activities of MIF. MIF(50-65) may therefore be a valuable tool to devise novel MIF-based therapeutic strategies.

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SHP2 SPECIFICALLY INHIBITS IL-6 SIGNAL TRANSDUCTION - SPECIFICITY OF SHP2 FOR ATTENUATING IL-6 SIGNAL TRANSDUCTION

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Interleukin-6 (IL-6) signals through a receptor complex composed of the IL-6Rα and the signal transducer gp130. Receptor dimerization leads to activation of the JAK/STAT pathway as well as the induction of the mitogen activated protein kinase cascade. The cytoplasmic tyrosine 759 of gp130 is known to mediate attenuation of IL-6 signaling. This tyrosine is part of a receptor module recruiting the protein tyrosine phosphatase SHP2 and the feedback inhibitor SOCS3.
Recently, we have analyzed the contribution of SHP2 to the negative regulation of IL-6 signaling in detail (Lehmann et al. 2003 J. Biol. Chem. 287, 661-671): The inhibitory function of SHP2 was demonstrated by increased IL-6 signaling in cells with a deletion of exon 3 (encoding amino acids 46-110) within the SHP2 gene. This mutation leads to expressing an N-terminal truncated SHP2 protein lacking the N-terminal SH2-domain. In these cells, IL-6 mediates enhanced STAT-activation and gp130-dependent gene induction suggesting an inhibitory role of SHP2 in IL-6 signaling. Furthermore, increased IL-6 signaling was also observed after expression of catalytic inactive SHP2 in wild type cells. Thus, mutation of endogenous SHP2 as well as overexpression of dominant negative SHP2 leads to enhanced signaling. Additionally, forced targeting of SHP2 to gp130 rescued inhibition of receptors lacking the inhibitory tyrosine 759. Since this approach mimics SHP2 receptor binding independent from recruitment of SOCS3 to the receptor, these results further confirm an inhibitory role of SHP2 for IL-6 signaling.
Here we analyze the specificity of SHP2 and give further evidence for the inhibitory role of SHP2 in IL-6 signal transduction. Our data indicate that beyond receptor recruitment the catalytic domain of SHP2 also contributes to the specificity of SHP2.

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THE MAPK ERK IS ESSENTIAL FOR CYTOKINE-INDUCED TRANSCRIPTIONAL ACTIVITY OF NFκB

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Background: Cytokines, e.g. IL-1β are believed to cause pancreatic β-cell destruction leading to Type 1 diabetes. IL-1β signals iNOS expression and β-cell death via the MAPK (ERK, JNK and p38) and NFκB pathways.
Aim: To determine if IL-1β-induction of MAPK-signaling affects NFκB activation in β-cells
Methods: Clonal β-cells were preincubated with inhibitors of ERK (PD098059), JNK (Tat-JBD) and p38 (SB203580) ± IL-1β. Effects of MAPK inhibition on IL-1β-mediated iNOS expression and degradation of the NFκB inhibitor protein, IκB, was examined by Western Blotting. NFκB DNA binding and induced gene transcription was examined by EMSA and gene reporter assays, respectively.
Results: Combined inhibition of ERK and p38 decreased iNOS production by 70%. MAPK inhibition did not affect the IκB degradation or NFκB DNA binding. However, inhibition of ERK reduced NFκB-mediated gene transcription by 60%.
Conclusion: ERK and p38 are essential for IL-1β-induced iNOS expression. Although cross-signaling between the MAPK and NFκB pathways was not observed at the level of DNA binding or upstream of this, ERK activity is required for NFκB gene transcription, strongly suggesting that ERK mediates regulation of iNOS expression via NFκB transactivating mechanisms.

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IL-7 INDUCES A MID TO LATE G_1 ACTIVATION OF PI 3-KINASE WHICH IS CRITICAL FOR CELL CYCLE ENTRY IN PRIMARY HUMAN T CELLS

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The PI 3-kinase pathway is thought to be essential for T cell growth and is potently activated by IL-2 within minutes. However another T cell growth factor, IL-7 failed to induce any activation of the pathway thus questioning a physiological role for PI 3-kinase in proliferation. We report here that IL-7- and IL-2-induced T cell proliferation are both sensitive to inhibitors of PI 3-kinase but also observed that IL-7 did activate the PI 3-kinase pathway 3 hours after stimulation. This activation was sustained up to 9 hours and is sensitive to cycloheximide. IL-2-induced activation is similarly sustained suggesting it is this phase of activation lasting through mid to late G_1 which is critical for cell cycle entry; results supported by our observation that addition of PI 3-kinase inhibitors up to three hours post-stimulation was effective at inhibiting proliferation. These studies also suggest that the late phase is indirect, requiring protein synthesis and highlight the differences in kinetics of PI 3-kinase activation by the γ_c T cell growth factors IL-2 and IL-7.

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STAT5 CONTRIBUTES TO T-CELL HOMEOSTASIS AND ONCOGENESIS

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STAT proteins are latent transcription factors that mediate a wide range of actions induced by cytokines, interferons, and growth factors. Whereas Stat5a$^{-/-}$ and Stat5b$^{-/-}$mice have decreased numbers of CD8$^+$ splenocytes, expression of either a Stat5a or Stat5b transgene increased the number of CD8$^+$ memory T cells. Consistent with a role for IL-15 in this expansion, the Stat5b transgenic mice demonstrated an increased proliferative response to IL-15, and this was enhanced following injection of Poly I:C. We also demonstrated increased AICD of CD4$^+$ T cells in Stat5 transgenic mice, possibly resulting from an increase in IL-2 signaling. Interestingly, we also noted the development of thymic T-cell lymphoblastic lymphomas in transgenic mice over time. The rate of lymphoma induction was markedly enhanced by immunization or by the introduction of TCR transgenes. Remarkably, the Stat5 transgene potently induced development of CD8$^+$ T cells, even in mice expressing a class II-restricted TCR transgene, with resulting CD8$^+$ T-cell lymphomas. The effect of Stat5 levels on CD8$^+$ memory T cell number indicate an important role for Stat5 in CD8$^+$ T-cell homeostasis. In addition, these data demonstrate the oncogenic potential of dysregulated expression of a STAT protein that is not constitutively activated, and that TCR stimulation can contribute to this process.

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TNF-INDUCED NF-κB ACTIVATION IS IMPAIRED IN FOCAL ADHESION KINASE (FAK)-DEFICIENT FIBROBLASTS

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Focal adhesion kinase (FAK) is widely involved in important cellular functions such as proliferation, migration, and survival. We demonstrate a critical role for FAK in the TNF-induced activation of NF-κB, using FAK-deficient (FAK-/-) embryonic fibroblasts. Interestingly, TNF-induced IL-6 production was nearly abolished in FAK-/- fibroblasts, while a normal production was obtained in FAK ± or FAK+/+ fibroblasts. FAK deficiency did not affect three types of MAP kinases, ERK, JNK and p38. Similarly, activation by TNF of neither AP-1 nor NF-IL-6 was impaired in FAK-/- cells. Of note, TNF-induced NF-κB DNA binding activity and activation of IκB-kinases (IKKs) were markedly impaired in FAK-/- cells, while the expression of TNFRI or other signaling molecules such as RIP, TRAF2, IKKα, IKKβ, and IKKγ was unchanged. Also, TNF-induced association of FAK with RIP and subsequent association of RIP with TRAF2 were not observed, resulting in a failure of RIP to recruit the IKK complex in FAK-/- cells. The reintroduction of wild type FAK into FAK-/- cells restored the interaction of RIP with TRAF2 and the IKK complex, and recovered NF-κB activation and subsequent IL-6 production. Thus, we propose a novel role for FAK in the NF-κB activation pathway leading to the production of cytokines.

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INDUCTION OF MAP KINASE/RHO PATHWAYS AND MODULATION OF SMAD FUNCTION IN TGF-β MEDIATED GROWTH ARREST OF BREAST CARCENOMA CELLS IN VITRO

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TGF-β signaling is mediated by Smad's, which are immediate downstream effectors of activated TGF-β receptor complex. In addition to the Smad signaling pathway, TGF-β also activates other pathways including the mitogen-activated protein (MAP) kinases ERK1/2, JNK, Rho and p38. These pathways often act together with Smad signaling to control gene expression and cell phenotype and deregulation of these pathways are likely to contribute to the pro-oncogenic activities of TGF-β.
We have used a cell system with defined oncogenic potential derived from the parental MCF10A human breast cell line to asses the role of MAP Kinase/Rho pathways on the function of Smad 2/3 in TGF-β mediated growth inhibition. MCF10CA1h cell line forms well-differentiated xenografted tumors and is growth inhibited up on TGF-β treatment and show activation of ERK/MAPKinase, p38 and Rho pathways apart from Smad2 and 3. Combined treatment of these cells with PD98059 (MEK inhibitor) or SB203580 (p38 inhibitor) or Y27632 (ROCK inhibitor) reduced the effect of TGF-β on cell growth in a significant manner, without altering the phosphorylation of Smad2/3, suggesting that these pathways may be mediating the anti-growth effect. A specific inhibitor of ALK5 (TβRI), SB431542 abolished the growth inhibiton suggesting that this effect is ALK5 dependent. Cells overexpressing Smad2 or Smad3 responded in a similar manner to those of wild type cells. However, cells expressing dominant negative Smad3 (smad3Δ C'), which inhibits phosphorylation of both Smad2 and Smad3, did not respond to TGF-β, suggesting that functionally active smad's are necessary for this process. Cells over expressing Smad3 as well as dominant negative Smad2/3 showed activation of ERK/MAP Kinase, p38 and Rho in comparable levels to those of wild type cells, suggesting that deregulation of Smad pathway did not effect the activation of these pathways. From these results, we conclude that MAP Kinase/Rho pathways are necessary for the growth inhibition of MCF10A cells, which in turn is achieved by modulating the function of Smad's. Currently, we are examining the effect of MAP Kinase/Rho inhibitors on the localization and DNA-binding ability of Smad2 and Smad3 with or without TGF-β treatment. We are also investigating the target genes downstream to these pathways.

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A PAN-PROTEIN KINASE C INHIBITOR DIFFERENTIALLY REGULATES TLR-3-MEDIATED SIGNALLING PATHWAY IN HUMAN DENDRITIC CELLS

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Dendritic cells (DC) express different Toll-like receptors (TLR) which recognize molecular patterns specific to microbial pathogens. During infection, signaling through TLR strongly activates DC to undergo a process of maturation which results in the upregulation of MHC and costimulatory molecules and the production of numerous cytokines that are important for initiating T-cell mediated immune responses. In the present study, we have investigated the role of PKC in the maturation of monocyte-derived DC by the viral double stranded RNA-like molecule poly (I:C), a specific ligand for TLR-3. TLR-3 engagement initiates a MyD88-independent cascade that culminates in the generation of Th1-inducing cytokines. The pro-inflammatory cytokine repertoire studied includes: TNF-α IFN-α IFN-β and IL-12p70. Inhibition of PKC activation by bisindolylmaleimide (Bis), a pan-PKC inhibitor, resulted in a decrease of poly (I:C)-induced bioactive IL-12p70 production while IL-12(p40) and TNF-α were not affected. We next paid particular attention to the influence of PKC inhibition on DC capacity to produce type I interferons which are crucial cytokines for anti-viral defense. We found that inhibition of PKC during poly (I:C) stimulation enhances IFN-α secretion by DC while decreasing IFN-β. Moving forward, we will investigate PKC inhibition effects on NF-κB, Stat 1 and IRF activation.

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DISRUPTION OF THE C-JUN-JNK COMPLEX BY A CELL-PERMEABLE PEPTIDE AFFECTS A DISTINCT SET OF IL-1-INDUCED INFLAMMATORY GENES

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The transcription factor activator protein (AP)-1 plays crucial roles in proliferation, cell death and the immune response. c-JUN is an important component of AP-1, but only very few c-JUN response genes have been identified to date. Activity of c-JUN is controlled by N-terminal phosphorylation (JNP) of its transactivation domain by a family of JUN-N-terminal protein kinases (JNK). JNK form a stable complex with c-JUN *in vitro* and *in vivo*. We have targetted this interaction by means of a cell-permeable peptide containing the JNK-binding domain of human c-JUN. This peptide strongly and specifically induced apoptosis in HeLa tumor cells, which was paralled by inhibition of serum-induced c-JUN phosphorylation and up-regulation of the cell cycle inhibitor $p21^{cip/waf}$. Application of the c-JUN peptide to IL-1-stimulated human primary fibroblasts resulted in up-regulation of six genes, namely COX-2, MnSOD, IkBalpha, MAIL, GM-CSF, and MMP-3 and down-regulation of nine genes, namely CCL8, mPGES, SAA1, hIAP-1, hIAP-2, pent(r)axin-3, CXCL10, ICAM-1, and CCL2. Only a small group of genes, namely pent(r)axin-3, CXCL10, ICAM-1 and IL-1beta was inhibited by both the c-JUN peptide and the JNK inhibitor SP600125. Thereby, we identify for the first time three distinct groups of inflammatory genes whose IL-1-induced expression depends on c-JUN, on JNK, or on both. These results shed further light on the complexity of c-JUN-JNK-mediated gene regulation and also highlight the potential use of dissecting signalling downstream from JNK to specifically target proliferative diseases or the inflammatory response.

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A ROLE FOR PROTEIN KINASE C IN REGULATING PROINFLAMMATORY SIGNALLING PATHWAYS IN PRIMARY HUMAN MONOCYTES

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Protein kinase C (PKC) isozymes constitute a family of 11 serine/threonine kinases whose substrate specificity is regulated via spatial and temporal targeting to different subcellular compartments. Localisation is effected via interaction with PKC isozyme-specific anchoring proteins called receptors for activated C kinase (RACKs). PKCs have been implicated in various regulatory signalling pathways controlling proliferation, activation and functional responses in immune cells, including macrophages.

Previous studies using non-isozyme selective pharmacological inhibitors implicated PKC in lipopolysaccharide (LPS)-stimulated secretion of TNFα by macrophages. However, such an approach lacks the discrimination of rationally designed inhibitors based on short PKC-derived peptides that mimic the ability of RACKs to modulate translocation and activation of PKCs in an isozyme-specific fashion.

We present data that supports a role for PKC in LPS-induced TNFα secretion, and highlights the potential of a recently described oligoarginine transporter system to deliver PKC isozyme-selective peptide inhibitors into primary human monocyte/macrophages.

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THE ROLE OF HCK TYROSINE KINASE IN CYTOKINE PRODUCTION BY HUMAN MACROPHAGES

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Src family tyrosine kinases (SFK) are among the first molecules activated following lipopolysaccharide (LPS) treatment, however their role in inflammatory cytokine production by human macrophages is unclear. Hck is a SFK produced exclusively by haematopoietic cells and previous studies using murine cell lines have shown a role for Hck in TNFα production. We show that Hck is expressed by both primary human monocytes and macrophages and that its expression is upregulated by 18 hours LPS treatment. Adenoviral constructs expressing wild type and kinase-inactive forms of Hck were used to examine the effect of their overexpression on cytokine production. Overexpression of Hck wild type resulted in a 4-6 fold increase in both TNFα and IL-6, whilst a 20-60 fold induction of IL-8 was observed for LPS-stimulated infected macrophages. There was no observable effect on IL-10 production. However, kinase-inactive forms had no effect on the cytokine production in this model. The related SFK, Src, also showed no effect on cytokine production by these cells. LPS-mediated IkBa degradation and NF-κB transactivation were unaffected in Hck wt infected macrophages. These data show that Hck has a role in LPS-induced proinflammatory cytokine production in human macrophages via a mechanism that does not require NF-κB.

TH1-TH2

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ALTERED CYTOKINE EXPRESSION OF WHOLE BLOOD T HELPER AND ALTERED CYTOKINE EXPRESSION OF WHOLE BLOOD T HELPER AND T CYTOTOXIC LYMPHOCYTES OF POLYMYOSITIS/DERMATOMYOSITIS PATIENTS

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Objective: To investigate the intracellular and soluble cytokine levels and T cell subsets in peripheral blood of patients with active and inactive polymyositis (PM) and dermatomyositis (DM).
Methods: The rates of T and B lymphocytes, T helper and T cytotoxic cells and the frequency of IFN-γ, IL-4 and IL-10 expression of $CD4^+$ or $CD8^+$ cells were determined by flow cytometry. We measured the concentrations of soluble cytokines with commercial ELISAs.
Results: In active DM (n = 29) we observed decreased T ($CD3^+$) and T cytotoxic ($CD8^+$) lymphocyte ratios, decreased IFN-γ expression of $CD4^+$ and $CD8^+$ cells, but a significantly increased B lymphocyte ratio. These prominent changes disappeared in the inactive stage (n = 20) of the disease. In PM (n = 50) we could not detect a significant change in these lymphocyte and T cell subsets. Both in DM and in PM we observed an increase in the number of IL-10 secreting cells, although it was significant only in inactive DM. We calculated the ratio of IL-4+/IFN-γ+ T helper cells and in active DM a significantly increased Th2/Th1 ratio, while in inactive DM a significantly decreased Th2/Th1 ratio was observed compared to the control population.
Conclusion: The most important finding in our work was the pronounced difference in the frequency of the peripheral blood lymphocyte subsets and also in the intracellular cytokine content of these cells between patients with active and inactive PM and DM.

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THE IMPACT OF DYDROGESTERONE SUPPLEMENTATION ON SERUM CYTOKINES CONCENTRATIONS AMONG WOMEN WITH THREATHENED ABORTION – THE PREELIMINARY REPORT.

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Objectives: The immunological pregnancy protective effect of progesterone could be manifested via controlling cytokines' production. The main aim of this prospective study was to compare serum concentrations of selected cytokines (TNF-alpha, IL-12 and IL-10) between women with threathened abortion (TA) and women with physiologic course of pregnancy and to evaluate the impact of dydrogesterone supplementation in women with TA on their cytokines' profile.
Methods: The study group comprised 30 pregnant women between 6 and 12 week's gestation with clinical signs of TA and 15 women with physiologic course of pregnancy (reference group). During I examination serum cytokines levels (TNF-alpha, IL-10 and IL-12) were measured using ELISA method (TECAN, Austria) in both groups. Treatment by dydrogesterone (20-40 mg/day) has been introduced in TA group only and after 10 days serum cytokines levels were evaluated again in both groups.
Results: Mean concentrations of evaluated cytokines did not differ significantly at I examination between the analyzed groups (8,01 pg/mL vs. 7,99 pg/mL in reference group for TNF-alpha; 3,99 pg/mL vs. 4,31 pg/mL for IL-12 and 7,40 pg/mL vs. 7,37 pg/mL; respectively). In II examination TNF-alpha concentration was higher in boths groups while IL-10 and IL-12 concentrations were llowered with no significant differences between the groups. TNF-alpha/IL-10 ratio changed from 1.11 to 1,72 while IL-12/IL-10 ratio remained the same (0,65 to 0,71), as compared between 2 examinations.
Conclusions: No significant differences in serum concentrations of Th1 and Th2-types cytokines was observed between women with threathened abortion as compare to normal pregnancy. Whether this effect, contrary to the results obtained among women with spontaneous abortion, might be explained by different immunity of TA or by dydrogesterone supplementation should be evaluated in further study.

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THE ADJUVANT PROPERTIES OF PERTUSSIS TOXIN AT THE T CELL LEVEL

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Pertussis toxin (PTX) is a major virulence factor of *Bordetella pertussis*, the agent of whooping cough. It consists of two moieties with different activities: the A subunit exerts its ADP-ribosyltransferase activity on the α-subunit of Gi proteins while the B oligomer allows the binding of PTX to target-cell receptors. Beside its toxic activity, PTX has also been described as an immunostimulatory factor in animal models and in humans. Furthermore, *in vivo* experiments demonstrated that PTX enhanced humoral and cell-mediated immune responses to co-administrated antigens.
The mechanisms by which PTX exerts its immunostimulatory properties are still unknown.
We first demonstrated that PTX efficiently induced maturation of human myeloid dendritic cells and IL-12 production. This activation was strongly impaired in neonatal myeloid DC (S.Tonon et al, *Eur.J.Immunol.*, 2002, 32:3118-3125).
We are now analyzing the effects of PTX on purified human $CD4^+$ T lymphocytes.
The majority of adult purified $CD4^+$T cells exposed to PTX without APC, underwent a dose-dependent upregulation of CD40L, CD69 and CD25 surface molecules as well as a homotypic aggregation. Moreover, PTX- stimulated $CD4^+$T cells were able to proliferate and to secrete significant levels of IFN-γ, IL-5, IL-10, IL-2 and TNF-α cytokines.
We next compared the response of adult naive $CD4^+RA^+$T and neonatal $CD4^+$T lymphocytes to PTX. Both T cells populations did proliferate and showed an increased upregulation of CD40L, CD69 and CD25 molecule expression in response to PTX. Nevertheless, the activation of neonatal $CD4^+$T lymphocytes in the presence of the toxin was clearly deficient, as compared to the adult T cells.
Our results thus suggest that the immunostimulatory properties of PTX could be related both to dendritic cell maturation, as well as $CD4^+$T cell activation.

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THE IMMUNOMODULATOR AS101 INHIBITS *IN-VITRO* AND *IN-VIVO* ACTIVITY OF THE INTERLEUKIN-10-LIKE CYTOKINE IN TILAPIA AND CARP FISH

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Outbreaks of opportunistic infections oppose a major threat to fish populations. These outbreaks are highly influenced by environmental and endogenous factors, which may cause opportunistic infections. Interleukin-10 (IL-10) is known in mammalians to down-regulate the cellular immunity, increasing the organism's susceptibility to opportunistic disease. We have previously shown the ability of the immunomodulator AS101 to regulate IL-10 levels in murines and humans. We report the finding of an IL-10-like (IL-10L) cytokine in Tilapia (*O. nil* X *O. aur* hybrid) and Carp fish (*Cyprinus carpio*) using Western-Blot analysis with human IL-10 mAb and ELISA. We compared the effects of LPS and PHA mitogens on intracellular *in-vitro* PMBC (Peripheral Mononuclear Blood Cells) IL-10L levels and show kinetics for both intracellular IL-10L synthesis in LPS activated PMBC cell cultures and *in-vivo* stress-induced IL-10L secretion to the serum. We show that AS101 is able to inhibit Tilapia PMBC IL-10L synthesis *in-vitro* with a dose dependant effect. Moreover, using an *in-vivo* air-exposure stress model we show that water-dissolved AS101 is able to decrease stress induced IL-10L secretion to the serum in a dose dependant effect without interference to the stress reaction. These findings indicate that AS101 may prevent opportunistic diseases in stressed fish.

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THE CHICKEN TH2 CYTOKINE GENE CLUSTER – IS IL-5 a PSEUDOGENE?

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The chicken immune system lacks several components of the Th2 response found in mammals (including classical eosinophils, IgG subclass switching and IgE), leading to the hypothesis that chickens lack, or have a reduced number of, Th2 cytokines. To identify the prototypic Th2 cytokines (IL-4, IL-5 and IL-13) at the genomic level we used a novel approach based upon conservation of synteny. These genes and those encoding IL-3 and GM-CSF are closely linked on human chromosome 5 and mouse chromosome 11. To clone the corresponding area of the chicken genome, chicken BAC libraries were screened with probes to non-cytokine genes whose mammalian homologues also lie within the cluster. Two contiguous BACs were sequenced and shown to contain the chicken homologues of IL-4, IL-5 and IL-13. Identity at the amino acid level with mammalian homologues was between 20-30 %. Structurally, the predicted proteins have the potential to form 4-α-helical bundles, as do all members of the IL-4 family in mammals. Whilst chIL-4 and chIL-13 are transcriptionally active, chIL-5 appears to be a pseudogene. Sequence skimming of a third contiguous BAC has suggested the presence of both IL-3 and GM-CSF.

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$CD4^+$ $CD25^+$ REGULATORY T CELLS CONTROL TH1 RESPONSES TO FOREIGN ANTIGENS INDUCED BY MATURE DCs IN VIVO

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Recent evidence suggests that, in addition to their well known stimulatory properties, dendritic cells (DCs) may play a major role in peripheral tolerance. It is still unclear whether a distinct subtype or activation status of DC exists which promotes the differentiation of suppressor rather than effector T cells from naive precursors. In this work, we tested whether the naturally occurring $CD4^+CD25^+$ regulatory T (Treg) cells may control immune responses induced by DCs *in vivo*. We characterized the immune response induced by adoptive transfer of antigen-pulsed mature DCs into mice depleted or not of $CD25^+$cells. We found that the development of MHC class I and class II-restricted IFN-γ-producing cells was strongly enhanced in the absence of Treg. By contrast, Th2 priming was downregulated in the same conditions. This regulation was independent of IL-10 production by DCs. Of note, splenic DCs incubated *in vitro* with Toll-like receptor ligands (LPS or CpG) activated immune responses which remained sensitive to Treg function. Our data further show that mature DCs induced higher cytotoxic activity in CD25-depleted recipients as compared to untreated hosts. We conclude that Treg naturally exert a negative feedback mechanism on Th1-type responses induced by mature DCs *in vivo*.

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DIFFERENTIAL REGULATION OF TH1/TH2 IN RELEVANT TISSUES FOR SEPSIS PATHOGENESIS WITH A *LIMULUS* ANTI-LPS FACTOR-DERIVED PEPTIDE INCREASES SURVIVAL IN GRAM-POSITIVE SEPSIS

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Severe sepsis and septic shock are important causes of death in intensive care units. Although Gram-negative infections were predominant in the 1960s, Gram-positive infections have increased in the past two decades and now account for about half of the cases of severe sepsis. In this study, we examined the effect of a *Limulus* anti-LPS factor (LALF)-derived peptide on lung and liver Th1/Th2 cytokine mRNA levels during a Gram-positive sepsis. We also examined the morphopathological changes observed in these organs during the disease. Mice challenged with a high dose of *Staphylococcus haemolyticus* showed a severe damage in lung, typical from ARDS. On the other hand, the liver of the challenged mice denoted accumulation of bacteria's particles in liver sinusoids, associated with a severe inflammation response given for high levels of tissue mRNA pro-inflammatory cytokines. The treatment with the peptide $LALF_{32\text{-}51}$ ameliorated the sepsis induced effect on lung and liver and increased the survival of mice in a dose and timing dependent manner. Pre-treatment with the peptide $LALF_{32\text{-}51}$ differentially regulates TNF-α, IFN-γ, IL-12p40, IL-2 and IL-10 mRNA levels in lung and liver of peptide treated-mice, and limits the systemic inflammatory response. These findings provide for the first time the effectiveness of a LALF-derived peptide during a Gram-positive sepsis. Modulating of Th1/Th2 pattern in tissues relevant for sepsis correlates with improved outcome of the disease as denoted by increased survival.

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DIFFERENTIAL MODULATION OF TH2 CYTOKINE PRODUCTION BY BACTERIAL TOXINS

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Cholera toxin (CT) enhances Th2 responses to co-administered antigens. Much of the evidence to date indicates that CT functions as an adjuvant by modulating dendritic cell activation. However, bacterial AB toxins also interact with T cells and CT has been shown to induce apoptosis of $CD8^+$ T cells. Here we have examined the influence of CT on cytokine production and intracellular signaling in $CD4^+$ T cells. We demonstrated that CT inhibited cytokine production by CD3-activated naïve T cells and IFN-γ production by Th1 clones, but enhanced IL-5 production by committed Th2 cells and type 1 regulatory T (Tr1) cell clones. CT directly induced the production of IL-5, but inhibited IL-2, IL-4, IL-10 and IL-13 from EL4 cells. Activation of IL-5 production by CT was associated with activation of the MAP kinases p38 and ERK. Incubation of CT-stimulated EL4 cells with specific inhibitors of p38 (SB203580) and ERK (PD98059) resulted in a dose dependent reduction in CT-induced IL-5 production. In contrast, an inhibitor of JNK (SP600125) enhanced IL-5 production. Our findings demonstrate that CT targets distinct signaling pathways for Th1 and Th2 cytokine production, but also suggest that distinct signaling pathways are employed for different cytokines secreted by Th2 cells.

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IL-31, A NOVEL CYTOKINE MADE BY ACTIVATED T CELLS, SIGNALS THROUGH A NOVEL HETERO-DIMERIC RECEPTOR COMPLEX EXPRESSED IN SKIN

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We have identified a novel four-helix bundle cytokine, designated IL-31, which binds and signals through a novel heterodimeric receptor composed of a gp130-like receptor (IL-31RA) and oncostatin M-receptor β (OSM-Rβ). The IL-31 cDNA contains an opening reading frame encoding a 164 amino acid precursor and a mature polypeptide of 138 amino acids. The gene structure of IL-31 is most closely related to LIF, oncostatin M (OSM) and Cardiotrophin. Northern blot analysis revealed low levels of IL-31 specific mRNA transcripts in bone marrow, peripheral blood mononuclear cells, testes and salivary gland. More importantly, the cDNA for IL-31 was isolated from an activated T cell library and is produced by activated T cells and NK cells. Semi-quantitative RT-PCR indicates that IL-31 is expressed early after T cell activation in predominantly CD4 + T cells and CD8 + T cells. IL-31 is preferentially expressed by Th2 cells, but is also produced (at lower levels) by Th1 T cells. IL-31 receptor can be induced in peripheral blood monocytes and skin fibroblasts, whereas epithelium from skin, lung and prostate express both receptors constitutively. IL-31 can induce STAT activation in cells expressing both IL-31R and OSMR-β and can induce IL-6, IL-8 and MCP-1 in some epithelial cell lines. Subcutaneous delivery of IL-31 induces alopecia and pruritis in BALB/c and C57Bl/6 mice. Histology of the regions of hairloss reveals hyperkeratosis, acanthosis and inflammation. Transgenic mice overexpressing IL-31 develop a severe pruritic skin phenotype resembling intrinsic atopic dermatitis. Taken together, these data indicate that IL-31 is a novel cytokine produced by activated Th2 skewed T cells and may play a role in promoting atopic skin diseases.

TNF FAMILY

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DELINEATION OF THREE SEPARATE IMMUNOGLOBULIN-BINDING SITES IN EACH C1q HEAD

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Crystallographic studies revealed that the TNF and the globular C1q module of mouse adipocyte complement related protein (mACRP30) have a closely related tertiary structure and trimeric organization. In this work the human C1q head conformation is designed using the atomic co-ordinates of mACRP30. The spectroscopic studies and modification with 2-oxy-5-nitrobenzylbromide show that all the three Trp residues in each human C1q head are exposed on the protein surface. Modification of the two less accessible Trp residues significantly affects the complement-dependent cytotoxicity (CDC). The pronounced effect of modification of the specific Trp and Arg residues on the CDC allows delineating three separate immunoglobulin-binding sites in each C1q head. The scanning calorimetry data reveal interacting globular domains in each human C1q head at the neutral pH region. Ionization or modification of His residues completely abolishes the interaction. This feature may regulate the activity of the classical complement pathway in the inflammation sites.

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TNF-α BINDS TO THE VARIANT SURFACE GLYCPROTEIN OF *TRYPANOSOMA CONGOLENSE*

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We recently showed that early mortality of mice experimentally infected with *T. congolense* or *T. brucei* is mediated by IFN-γ (Shi et al. Eur. J. Immunol. 2003. 33:108). There is ample evidence in the literature that TNF-α is a major mediator in the pathogenesis of African trypanosomiasis. Although we were able to show the presence of many cytokines in the plasma of infected mice by ELISA method, we failed to demonstrate a consistent presence of TNF-α. We hypothesized that TNF-α might bind to soluble variant surface glycoprotein (sVSG). sVSG has been shown to be present in the plasma of infected mice. Using ELISA, we very recently demonstrated that sVSG very effectively inhibited the binding of the detection antibody to TNF-α. Using similar techniques, we showed the complex of sVSG and TNF-α *in vivo*. The binding of TNF-α to sVSG has important implications in the pathogenesis of the disease.

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HIGH FREQUENCY OF TNF-α ALLELES -238A AND -376A IN SARDINIA

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The G to A single nucleotide polymorphisms (SNPs) at position – 376- and – 238 in the promoter of the tumor necrosis factor alpha (TNF-α) gene have been independently correlated with numerous diseases. Alleles TNF_{-376A} and TNF_{-238A} are considered rare as they are normally found throughout the world with very low frequencies even when a correlation with a disease was found. We report that the frequency of TNF_{-376A} and TNF_{-238A} is remarkably elevated in individuals from the island of Sardinia (0.32 and 0.36, respectively, with over 56% of the subjects tested carrying these alleles) as compared to Sicily (0.03 and 0.06), where it is similar to other populations throughout the world. Such elevated frequency may be the result of a selective pressure on TNFA or on neighboring genes, including the HLA genes, or of genetic drift. This finding indicates that Sardinia is an ideal location to elucidate further the correlation between TNF-α SNPs and the diseases which they have been associated. These diseases include malaria, endemic in Sardinia until the end of the 1940s, as well as multiple sclerosis and type I diabetes, two autoimmune diseases present in this island with an unusually high frequency and co-morbidity. (Sponsored by Novartis Foundation).

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ROLE OF CELL-SPECIFIC TNF PRODUCTION IN THE RESISTANCE AGAINST PATHOGENS REVEALED BY CONDITIONAL GENE TARGETING

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TNF plays a critical role in host defense and maintenance of secondary lymphoid tissues. TNF is produced in vivo by various cell types, including cells of both hematopoietic and non-hematopoietic origin. In order to evaluate the specific contribution of different cell types into pleiotropic TNF functions, we generated mice with "floxed" configuration of the TNF gene and then crossed them to $MLys^{Cre/Cre}$, $CD19^{Cre/Cre}$ and lck-Cre mice. As a result, TNF gene was deleted in macrophages/granulocytes or in B or in T cells. Phenotypic analysis revealed distinct and specific functions of TNF derived from each of these cell types. Several cell types contributed to systemic TNF production in response to LPS. However, deletion of TNF gene in macrophages only was sufficient to prevent lethal shock induced by LPS/D-Gal. Interestingly, all strains with tissue-specific TNF inactivation showed distinct phenotype in Listeria model. In addition, deletion of TNF in lymphocytes resulted in abnormal immunoglobulin responses and defects in secondary lymphoid tissues.

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DISTINCT RECOGNITION MOTIFS IN LTβR vs CD40 FOR TRAF3 SIGNALING

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Lymphotoxin-β receptor (LTβR) and CD40, are members of the tumor necrosis (TNF) family of signaling receptors that regulate cell survival or death through activation of NF-κB. These receptors transmit signals through downstream adaptor proteins called TRAFs (TNFR-associated factors). The crystal structures of a series of TNF signaling molecules have been determined, each in complex with TRAF3. Comparison of TRAF3 bound to LTβR, CD40, and the downstream regulator TANK, showed that the molecules bind to the same binding crevice on TRAF3. The crystal structure of the cytoplasmic domain of LTβR revealed an unexpected new recognition motif IPEEGD that is distinct in sequence and structure from the PVQET motif in CD40 and PIQCT in TANK. The analysis revealed structurally adaptive 'hot spots' in the TRAF3 crevice that provide a gallery of distinct molecular interactions required for specific signaling events. Numerous TNF receptors engage TRAFs. Thus the formation of each complex has important consequences. The adaptability defined in this study provides molecular insight into the complex cellular milieu of TRAF-mediated signaling.

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TRAIL-RESISTANT CELLS SENSITIZED TO APOPTOSIS BY SELECTIVE DOWN REGULATION BY siRNAs TO INHIBITORS OF APOPTOSIS BCL-2, FLIP, SURVIVIN OR XIAP.

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TRAIL/Apo2L shares homology in its extracellular domains with members of the tumor necrosis factor family. However, melanoma cells were relatively resistant to apoptosis by TRAIL /Apo2L. We postulated that resistance might result from expression of bcl-2, FLIP (an inhibitor of caspase 8) or intracellular inhibitors of apoptosis (IAPs) such as XIAP or survivin that result in suppression of caspase 3 actions. Lipofectamine-mediated transfection of resistant A375 melanoma cells with siRNAs (20-40 nM) to each of these apoptosis-modulating genes was 60-90% effective at 48 or 72 hrs in inhibiting gene product expression without effects on IFN-stimulated genes (STAT1, ISG15). No significant apoptosis (< 10% assessed both by TUNEL and Annexin V/PI staining) was evident at 60-72 hrs in A375 cells after treatment with lipofectamine, TRAIL (100 ng/ml), or siRNAs alone. When TRAIL was added for 16-24 hrs after 48 hrs of treatment with each siRNA, marked increases in apoptosis resulted with overall apoptotic cell frequencies of 40-70%. TRAIL alone resulted in 5-15x increase of caspase 8; transfection with each of the siRNAs further augmented caspase 8 by 3-6x. An apoptotic protease, poly ADP-ribose polymerase (PARP), downstream of caspase 3, was not activated by TRAIL alone. However, after transfection with each of the siRNAs, PARP cleavage resulted. Analogous results were obtained in a renal carcinoma, SK-RC45 assessed similarly. Thus, TRAIL resistance was overcome in melanoma and renal carcinoma either by interfering with expression of modulators of mitochondrial membrane integrity (bcl-2) or initiator and executioner caspases (IAPs).

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THE *TNF* REGION IN COELIAC DISEASE

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Aim: The aim of this study was to investigate the role of the *TNF* region in susceptibility to coeliac disease.
Methods: Three SNPs, and five microsatellite polymorphisms in the TNF region were analysed in 100 healthy controls and 120 treated coeliac patients (on gluten-free diet).
Results: Significant associations between these *TNF* polymorphisms and coeliac disease were found. Strongest associations were observed for the *TNF-308-2, TNFB1, TNFb3* and *TNFd1* alleles individually (OR > 4.0 for each). We also investigated TNF-α production by PBMC from these controls and patients. TNF-α production was significantly higher by resting and activated PBMC from patients compared to controls ($p < 0.02$ for all conditions tested). However, no direct relationship between TNF-α levels and *TNF* polymorphisms was found. Haplotype analysis demonstrated that the coelaic associated haplotype extends right through the TNF region. The most common haplotype in patients was the combination of *TNFa2, TNFb3, TNFc1, TNfd1, TNFe3, TNF-308-2, TNF-238-1, TNFB-1*, termed the 'TA' haplotype. This haplotype is present in 14.4% of controls compared to 50.0% of patients, $p < 0.001$. When *HLADQB1*0201* was included in the analysis, this 'TA + 0201' haplotype was present in 10.0% of controls compared to 32.8% of patients, $p < 0.006$. Interestingly, some of the *TNF* alleles on the 'TA + 201' haplotype appear to be inherited independently of the **0201* allele. The 'TA' haplotype was also present in 17.2% of patients who did not carry **0201*.
Conclusion: These results support the view that a site in or near the *TNF* region may contribute to the risk of developing coeliac disease.

TOLL-LIKE RECEPTORS

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TOLL-LIKE RECEPTOR (TLR)-4 ACTIVATES IRF-3 IN A COMPLEX WITH THE NF-κB SUBUNIT p65

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The interferon regulatory factor (IRF)-3 is a key transcription factor in the innate host response to bacterial lipopolysaccharide (LPS), which is sensed by the Toll- like receptor (TLR)-4, or to viral infection, which is largely driven by TLR3. Upon activation it binds to the interferon-stimulated response element (ISRE) to induce transcription of a subset of chemokines and cytokines including interferon-beta (IFNbeta). Virus- induced activation of IRF-3 has been examined in detail, culminating in the determination of critical C- terminal phosphorylation sites and the recent identification of an activating kinase complex comprising IKKε and TBK-1. LPS/TLR4- signalling to IRF-3 however employs a different pathway, which is IKKε– independent and does not lead to phosphorylation of the critical C- terminal residues.
Here we have examined the TLR4 signalling to IRF-3 and compared it to TLR3. We have found that inhibitors of NF-κB block LPS-induced activation of IRF-3 and that p65-deficient mouse embryonic fibroblasts (MEFs) are impaired in TLR4- signalling to the ISRE. TLR-3 signalling to IRF-3 however was neither affected by the NF-κB inhibitors nor by the loss of p65. We also show that p65 promotes the transactivation function of a Gal4-IRF-3 fusion protein and find that IRF-3 interacts with p65 endogenously in unstimulated cells. Upon stimulation with LPS, p65 was detected in the IRF-3 complex at the ISRE. Our study therefore provides a mechanism for IRF-3 activation by LPS, identifying a novel role for p65 and the NF-κB signalsome.

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TOLL-LIKE RECEPTOR 2 MEDIATES LUNG INFLAMMATION INDUCED BY NON-MANNOSE-CAPPED LIPOARABINNOMANNAN

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Non-mannose-capped lipoarabinomannan (AraLAM) is part of the cell membrane of atypical mycobacteria. To determine the capacity of AraLAM to induce lung inflammation *in vivo*, and to determine the signaling receptors involved herein, wild type (WT) mice, Lipopolysaccharide Binding Protein deficient mice (LBP KO), Toll-like receptor (TLR) 4 mutant mice or TLR2 KO mice were intranasally inoculated with purified AraLAM. AraLAM induced high lung levels of TNF, IL-1β, IL-6 and KC and an influx of neutrophils into the pulmonary compartment of WT mice; LBP KO and TLR4 mutant mice displayed similar inflammatory responses, whereas in TLR2 KO mice AraLAM-induced lung inflammation was strongly diminished. However, TLR2 KO mice displayed an unremarkable host defense against pulmonary infection with the atypical (AraLAM expressing) *Mycobacterium (M.) smegmatis,* as reflected by bacterial loads that were similar to the bacterial burden in WT mice; TNF levels and neutrophil numbers even were higher in lungs of TLR2 KO mice. TLR4 mutant mice were indistinguishable from WT mice during *M. smegmatis* infection. These results point out that TLR2 is an important signaling receptor of AraLAM in lungs *in vivo*. Nonetheless, TLR2 signaling is not crucial in host defense against fast growing mycobacteria expressing AraLAM.

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DUAL ROLE OF TOLL-LIKE RECEPTOR (TLR)4 IN SEPTIC PERITONITIS IN MICE: PROTECTION BY TLR4 DURING INITIAL INFECTION IS LOST WHEN PERITONITIS COMPLICATES ACUTE PANCREATITIS

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Pancreatitis is frequently complicated by Gram-negative infections. TLR4 plays a major role in the antibacterial host defense against Gram-negative infection; however, little is known about the role of TLR4 during infections complicating critical illness such as pancreatitis. We determined the influence of pancreatitis on host response to *E. coli* induced peritonitis in TLR4 competent and deficient mice.
Wild type and TLR4 mutant mice received $5*10^4$ CFU *E. coli* i.p., preceded by 12 hourly injections of either cerulein or saline (sham). TLR4 deficient mice subjected to peritonitis displayed a reduced ability to clear *E. coli* (more CFU in peritoneal lavage fluid, liver and blood ($P < 0.05$)). In separate experiments, pancreatitis developed comparably in Wt and TLR4 mutant mice. When peritonitis was preceded by acute pancreatitis, there was a marked decrease in host defense in Wt mice (CFU in PLF, blood and liver all higher in pancreatitis/peritonitis mice, $P < 0.05$ vs sham/peritonitis); however, this decreased host defense was absent in pancreatitis/peritonitis TLR4 mutant mice.
Therefore: 1. TLR4 is important for host defense against *E. coli* induced septic peritonitis. 2. Pancreatitis reduces host defense to septic peritonitis in Wt mice. 3. The role of TLR4 in host defense against septic peritonitis is lost when this infection complicates pancreatitis.

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EXPRESSION OF TLRS, ADAPTER MOLECULES AND INFLAMMATORY MEDIATORS DURING SUBLETHAL INFECTION WITH *S. ENTERICA*.

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Toll-like receptors (TLRs) selectively recognise pathogen associated molecular patterns (PAMPs) produced by either Gram-negative or Gram-positive bacteria to initiate an innate immune response. We have investigated the expression of a range of TLRs, adapter molecules such as MD-2, MyD88 and TIRAP/Mal and inflammatory mediators (iNOS, TNFα) by real time PCR in C57/BL6 mice. The mice were infected with *Salmonella enterica* Typhimurium (*S. typhimurium*) M525P leading to a sub lethal infection whereby the bacterial numbers were plateauing from day 4-7 onwards. In general higher basal RNA levels were observed in spleen compared to liver. During infection expression levels in spleen did not change very much. In contrast in liver the RNA levels of TLR-1, TLR-9, iNOS and TNFα show a large increase in response to infection. TLR-2 mRNA is strongly but transiently induced. TLR-4 mRNA levels also show a transient increase but less and delayed compared to TLR-2.

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RESPONSE OF BONE MARROW DERIVED MACROPHAGES TO *SALMONELLA ENTERICA* INFECTION

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Macrophage activation by salmonellae is likely to involve cellular recognition of a number of pathogen associated molecular patterns (PAMP). Using *Salmonella enterica* Typhimurium (*S. typhimurium*) M525P which leads to sub lethal infection in mice, the response of bone marrow derived macrophages will be investigated in more detail. *S. typhimurium* M525P infection leads to the expression and release of inflammatory mediators such as nitric oxide and various pro-inflammatory cytokines. As expected the mRNA levels of TLR-2 increase rapidly and transiently during infection. However the mRNA levels of other TLRs and adapter molecules investigated, did not change during the course of infection.

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DIFFERENTIAL ROLE OF TOLL/IL-1 RECEPTORS (TIRS) IN PROMOTING THE STABILIZATION OF ARE CONTAINING MRNAS

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IL-1 and LPS are known to promote stabilization of a subset of short-lived mRNAs containing AU rich elements (AREs) in their 3'UTRs via IL-1R1 or TLR4,.both members of the Toll/IL-1 receptor (TIR) family. It is now evident however, that different TIRs promote distinct biologic responses and stimulate distinct intracellular signaling events. Using HEK293 cells we observed that signals initiated through IL-1R1 or TLR4 but not TLR3 can promote the stabilization of several unstable chemokine mRNAs (e.g., IL-8, Gro, KC). This response required the ARE motif contained in the 3'UTR of the target gene as demonstrated using chimeric reporter constructs whose transcription is controlled through a tetracycline regulated promoter. Both TLR4 and IL-1R1 require the adapter protein myd88 and the IL-1 receptor associated kinase (IRAK) for signaling while TLR3 responses are independent of IRAK and use a distinct adapter molecule (TRIF or TICAM) in place of myd88. IRAK deficient cells retained some ability to stabilize target mRNAs in response to IL-1 and over-expression of IRAK, though capable of stimulating activation of NFκB, could not stabilize chemokine mRNA. In contrast over-expression of myd88 did promote stabilization of ARE-containing mRNA. These findings suggest that TIR signaling for the stabilization of mRNA depends upon signals initiated through the action of myd88 but are independent of IRAK.

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CHLAMYDIA INCREASES INTERFERON-GAMMA RECEPTOR EXPRESSION THROUGH TOLL-LIKE RECEPTOR SYSTEM

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Interferon-gamma (IFNγ) induces indoleamine dioxygenase (IDO), which effectively inhibits the growth of intracellular *Chlamydia* in vitro. *Chlamydia* significantly increases expression of the IFNγ receptor (IFNγR) in HeLa cells even when *Chlamydia* is inactivated, suggesting that chlamydial antigen and not infection up-regulates cytokine receptor expression. This study addresses the hypothesis that *Chlamydia* increases IFNγR expression through activation of the Toll-like receptor (TLR) system. HEK cells expressing only TLR2, TLR4/MD2 or no TLR were infected with *C. psittaci*. After 24 h, the cells were stained with anti-chlamydial LPS and anti-IFNγR antibodies and analyzed by two-color flow cytometry. Cells not possessing TLRs did not increase expression of cytokine receptor while those possessing either TLR2 or TLR4/MD2 increased IFNγR expression two-fold and 1.6-fold, respectively. As increases in IFNγR were greater with cells expressing TLR2 than with cells expressing TLR4/MD2, this suggests that the chlamydial antigen interacts more strongly with TLR2.

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TOLL-LIKE RECEPTORS – ARE THEY POTENTIAL THERAPEUTIC TARGETS IN RHEUMATOID ARTHRITIS?

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Rheumatoid arthritis (RA) is a chronic inflammatory disease of the joints. It is characterised by the presence of an inflammatory synovitis accompanied by destruction of joint cartilage and bone. Increased amounts of cytokines and chemokines have been detected in the RA synovium and joint damage is observed due to the release of metalloproteinases from activated fibroblasts and macrophages. Inhibition of the production of these factors could prove beneficial therapeutically in this disease. Recent evidence suggests that the innate immune system is implicated in the initiation of RA. Toll-like receptors have an essential role in the innate immune system and have been suggested as possible therapeutic targets in RA. Here we have examined expression of TLRs 1, 2, 3 and 4 in rheumatoid arthritis synovial membrane cells and functional inhibition using neutralising antibodies. Expression of these TLRs on the macrophage population was found to be either extremely low or not detectable. Neutralising antibodies that can inhibit primary macrophages stimulated *in vitro* were unable to block release of IL-6, IL-8 or TNF in rheumatoid arthritis synovial membrane cells.

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TOLL-LIKE RECEPTOR 4 CONTRIBUTES TO A PROTECTIVE IMMUNE RESPONSE TO *KLEBSIELLA* PNEUMONIA IN PREVIOUSLY HEALTHY MICE, BUT NOT IN MICE WITH A PREEXISTING ACUTE PHASE PROTEIN RESPONSE

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Background: TLR4 has been implicated in the innate immune response to Gram-negative bacteria. *Klebsiella pneumoniae* is a Gram-negative respiratory pathogen that especially afflicts hospitalized patients.
Objective: To determine the role of TLR4 in host defense against *Klebsiella* pneumonia in previously healthy mice, and in mice with a sterile acute phase protein response (APR) (such as commonly observed in patients with preexisting disease).
Methods: TLR4 mutant C3H/HeJ mice and normal wild type (WT) C3H/HeN mice were injected subcutaneously with turpentine (to induce an APR) or saline (control) in both hind limbs two days before intranasal inoculation with *K. pneumoniae*.
Results: Turpentine-injected mice demonstrated weight loss and an APR ($P < .05$ vs saline). Pulmonary bacterial clearance was impaired in control TLR4 mutant compared to WT mice ($P < .05$). In contrast, TLR4 mutant mice with an APR displayed bacterial loads that were similar to WT mice with an APR. An APR was associated with reduced cytokine and chemokine levels, and a reduced myeloperoxidase activity in lungs when compared to control mice, regardless of the presence or absence of a functional TLR4.
Conclusion: In the presence of an APR, TLR4 loses its protective role in host defense against *K. pneumoniae* pneumonia.

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NOD2 MEDIATES INDUCTION OF THE ANTIINFLAMMATORY CYTOKINE IL-10 BY TLR2-LIGANDS: RELATION WITH CROHN'S DISEASE

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Nod2 is an important susceptibility gene in Crohn's disease. However, no pathogenetic explanation for this association is available. Using PCR and restriction fragment length polymorphysm analysis we have identified four Crohn's disease patients homozygous for the 3020insC frameshift mutation. When mononuclear cells (MNC) from these patients were stimulated with LPS, production of TNF, IL-6 and IL-10 was similar compared to six Crohn's patients without the mutation and six healthy volunteers. In contrast, when MNC from the patients were stimulated with the TLR2 agonists peptidoglycan and Pam3Cys lipopeptide, a severely impaired IL-10 (10 to 20% of normal production, $p < 0.01$), but not TNF and IL-6 release, was found. *Bacteroides* species from the intestine have been suggested to induce inflammatory reactions in Crohn's disease. Stimulation of cytokine production by *B. fragilis* was TLR2-dependent and TLR4-independent, as we demonstrate in knock-out mice. MNC of Crohn's patients with the Nod2 polymorphysm produced significantly less IL-10 upon stimulation with *B. fragilis* (25 to 40% of normal production, $p < 0.03$). In conclusion, Nod2 is essential for the release of IL-10 induced by TLR2 agonists in the intestinal tract, and this is the first mechanism to explain the increased susceptibility to Crohn's disease in patients with Nod2 mutations.

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TOLL-LIKE RECEPTOR-2 INHIBITS CELLULAR RESPONSES AGAINST *CANDIDA ALBICANS* THROUGH PATHWAYS MEDIATED BY IL-10 AND CD4 + CD25 + REGULATORY T CELLS

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Toll-like receptor (TLR)-2 and TLR4 have been shown to be involved in recognition of *Candida albicans*. Surprisingly, TLR2 knock-out (–/–) mice were more resistant to disseminated *C. albicans* infection compared to control mice: survival of TLR2–/– mice was 40% compared with 0% in TLR2+/+ mice after a lethal *Candida* challenge; fungal load 10- to 20-fold lower in the organs of TLR2–/– mice. This was associated with an increased chemotaxis and enhanced candidacidal capacity of TLR2–/– macrophages. The increased resistance of TLR2–/– mice to disseminated candidiasis is due to severely impaired IL-10 production, whereas the release of proinflammatory cytokines TNF and IL-1 was normal. Moreover, TLR2–/– mice showed a 50% decrease in the $CD4^+$ CD25 + regulatory T (Treg) cell population, and in-vitro experiments demonstrated induction of the survival of Treg cells by TLR2 agonists. The deleterious role of Treg cells on the innate imune response during disseminated candidiasis was demonstrated by the improved resistance to disseminated candidiasis after specific depletion of Treg cells. In conclusion, TLR2 mediate IL-10 production and Treg survival, and *C. albicans* induces immunosuppression through these mechanisms to escape the immune system. This represents a novel pathogenetic mechanism in fungal infections.

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THE ROLE OF BRUTON'S TYROSINE KINASE IN CYTOKINE PRODUCTION BY HUMAN MACROPHAGES

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Bruton's tyrosine kinase (Btk) is a member of the Tec family of tyrosine kinases. Deficiency of Btk results in immunodeficiency (X-linked agammaglobulinemia, XLA) in humans, a disease characterised by low amounts of B cells and immunoglobulins. Previously, we have shown that Btk-deficient peripheral blood mononuclear cells from XLA patients have impaired TNFα to TLR-4 ligand bacterial LPS. Additionally, overexpression of Btk in normal human macrophages results in enhanced TNFα production, to LPS, by stabilisation of TNFα mRNA (JEM in press). We have now extended our studies to examine a number of inflammatory cytokines and different stimuli. Investigation of cytokine responses using TLR-2 ligands, zymosan and Pam-3-cis, in XLA patients has shown that TNFα and IL-1β responses are severely abrogated. Other cytokines, IL-6, IL-8 and IL-10 were unaffected in XLA patients when PBMC were activated with either LPS or Pam-3-cis. However, IL-6 and IL-8 were severely reduced in XLA patients stimulated with zymosan. Similarly, overexpression of Btk in normal macrophages resulted in enhanced TNFα production to LPS, zymosan and Pam-3-cis but had no effect on other cytokines namely IL-6, IL-8 and IL-10. These results show that Btk has an important role in signalling of both TNFα and IL-1β, in response to a broad range of TLR ligands.

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TLR3 SELECTIVELY SIGNALS VIA MAL AND IKKβ TO INDUCE NFκB ACTIVATION AND CYTOKINE PRODUCTION IN HUVEC

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Toll-like receptors (TLR) are innate immune recognition molecules that are essential in identification of microbial products. Amongst these, TLR3 is described as the receptor for double-stranded (ds)RNA, a molecular pattern associated with viral infections. The signalling pathways activated through specific TLRs are being elucidated and differences are starting to emerge, where responses specific for distinct TLRs are dependent on the regulation of a set of adaptor molecules. We have investigated TLR3 signalling in human umbilical vein endothelial cells (HUVEC) and dendritic cells (DC). Here we demonstrate that the responses initiated from TLR3 stimulation are cell type dependent. We observed in HUVEC that TLR3 specifically uses the adaptor molecule Mal (MyD88-adaptor-like) to regulate cytokine production in a NFκB and IKKβ (IκB kinase) dependent pathway. This is in contrast to previous studies using the HEK293 cell line, where the adapter molecule TRIF (TIR domain-containing adapter inducing IFN-beta) and IKKε were suggested to control this pathway. By contrast, in DC we show that, although TLR3 induces IFN-inducible genes, this is independent of NFκB and IRF-3 in a pathway that appears not to be controlled by Mal. Our results provide new insight into the molecular mechanisms leading to human cell activation during viral infection.

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TOLL-LIKE RECEPTOR 4 CONTRIBUTES TO THE EFFICIENT CONTROL OF INFECTION WITH THE PROTOZOAN PARASITE *LEISHMANIA MAJOR*

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The essential role of Toll like receptors (TLR) in innate immune responses to bacterial pathogens is increasingly recognized, but very little is known about the role of TLRs in host defence against infections with eukaryotic pathogens. In the present study, we investigate whether TLRs contribute to the innate and acquired immune response to infection with the intracellular protozoan parasite *Leishmania major*. Our results show that TLR4 contributes to the control of parasite growth in both phases of the immune response. We also address the mechanism that results in killing or growth of the intracellular parasites. Control of parasite replication
correlates with the early induction of inducible nitric oxide synthase in TLR4 competent mice whereas increased parasite survival in host cells from TLR4 deficient mice correlates with higher activity of arginase, an enzyme known to promote parasite growth. This is the first study showing that TLR4 contributes to the effective control of Leishmania infection *in vivo*.

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REGULATION OF THE HUMAN *TRIF* GENE

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Our understanding of the mechanisms by which the adaptor proteins for the Toll-like receptors (TLRs) facilitate signaling in response to pathogen-associated molecular patterns (PAMPs) such as LPS is growing at a rapid pace. In comparison, however, the regulation of these adaptor proteins (which currently include Mal/TIRAP, MyD88, TRIF/TICAM-1, SARM/DRP and TIRP/TICAM-2) at the genomic level is poorly understood. In order to address this deficiency, we have used promoter-deletion reporter constructs to perform a detailed annotation of the human *TRIF* gene and fine analysis of the basal and inducible promoter elements lying 5' to the site of initiation of transcription. We identified a distal region with the ability to negatively regulate basal transcription and a proximal region that confers approximately 70% of the basal transcriptional activity. The human *TRIF* gene can be induced by over-expression of multiple stimuli. Induction of *TRIF* by TRIF itself, TLR3, IRF3, LPS, TNFα and IL-1 all require a distal NFκB element, while induction by TLR4 and Mal and MyD88 appear to require a more proximal element. *TRIF* would therefore appear to be dynamically regulated by a large number of stimuli, emphasising its importance as a regulator of innate immunity.

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THE MURINE IRAK2 GENE GENERATES 4 SPLICE VARIANTS, ONE OF WHICH IS INHIBITORY

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IRAK-2 is a protein that participates in signal transduction by the IL-1 receptor/Toll-like receptor (TLR) superfamily. We have performed detailed genomic analysis of the murine *Irak2* gene. *Irak2* is alternatively spliced to generate 4 different natural isoforms with potentially diverse functions. The full-length protein has been designated Irak-2a; another, called Irak-2b, is missing a region between the putative death and kinase domains whose function is still unknown. A third isoform (Irak-2c) lacks the entire N-terminus and the fourth (Irak-2d) lacks both the death domain and a small region at the C-terminus. The *Irak-2c* mRNA is also distinguished by its own 5' UTR and promoter elements, suggesting a distinct function. Functional studies showed that Irak-2c behaved as a dominant negative in comparison to Irak-2a, -2b and -2d, which all ectopically induced NFκB-luciferase expression and enhanced the expression of LPS-induced NFκB activity in murine NIH3T3 cells. Interestingly, no evidence of alternate human *IRAK-2* variants was found, suggesting that the IL-1R/TLR system is differentially regulated in humans and mice. Surprisingly, full-length murine Irak-2 acted as an inhibitor of TNFα-induced NFκB, while human IRAK-2 acted as a potentiator, further suggesting that these orthologues have divergent functions despite a high degree of amino acid identity.

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PI3K AND NEGATIVE REGULATION OF TLR SIGNALING

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Although Toll-like receptors (TLRs)-mediated interleukin 12 (IL-12) production by dendritic cells (DCs) confers protection against harmful invasions by regulating both innate and adaptive immunity, its dysregulation may lead to detrimental effects on hosts. We demonstrate in this study that phosphoinositide 3-kinase (PI3K) negatively regulates TLRs-induced IL-12 synthesis by DCs. TLRs stimuli inducing IL-12 production concomitantly elicit PI3K activation in DCs, but both $PI3K^{-/-}$ and PI3K inhibitor-treated DCs demonstrated increased IL-12 production. Consistently, activations of several signal transduction pathways triggered by TLRs (including p38 mitogen activated protein kinase pathway) are enhanced in such DCs with low PI3K activity. As the physiological consequence, enhanced T helper cell type 1 (Th1) response and a healing phenotype were observed upon *Leishmania major* infection in $PI3K^{-/-}$ mice on a *L. major*-sensitive BALB/c background. Our findings suggest the presence of negative regulatory mechanisms involving PI3K for TLR signaling during innate immune activation that likely contribute to prevention of excessive Th1 polarization causing undesirable immune responses.

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TOLL-LIKE RECEPTOR 10 IS EXPRESSED BY HUMAN B CELLS AND PLASMACYTOID DC, AND HAS NO FUNCTIONAL EQUIVALENT IN MOUSE

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Toll like receptors (TLRs) represent a family of mammalian proteins involved in responses to pathogen-derived products. 10 TLRs are described for human and rat, but only 9 for mouse. We present evidence for the lack of TLR10 in mouse due to a retroviral insertion in the coding portion of this gene; a presumably functional TLR10 gene is present in rat. TLR10 is part of a gene locus which also contains TLR1 and TLR6, closely related proteins known to associate with TLR2 for ligand recognition.
We present evidence that TLR10 in human is expressed by Plasmacytoid dendritic cells and B cells. Northern blot shows expression only in immune tissues, with highest expression in lymph node and spleen. TLR10 is a heavily N-glycosylated protein of 90-100kDa, as demonstrated by transfection of a HA-tagged clone, and Western Blot of B cell lines. We have demonstrated associations between TLRs expressed in B cells and Plasmacytoid dendritic cells, but not MD-1 or MD-2, by co-immunoprecipitation. Mutants in a TLR10-CD4 fusion protein abolished activation of luciferase reporters containing cytokine gene promoters.
Human TLR10 represents a functional member of the TLR family and may contribute to innate immune responses mediated solely or in conjunction with other TLRs.

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PI3-KINASE INHIBITION RESULTS IN THE HYPER-RESPONSIVNESS OF DENDRITIC CELLS TO LPS AND DS RNA POLY(I:C)

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Distinct negative regulatory mechanisms within the innate immunity exist to achieve controlled activation of the immune responses enabling protection from detrimental effects of an exacerbated or overshooting reacrions.
Herein, we provide evidence that phosphoinositol-3 kinase (PI3K) is a negative feedback regulator of LPS and double stranded (ds) RNA poly(I:C) specific signaling pathways which limits the production of inflammatory mediators in monocyte-derived dendritic cells (DCs). Inhibition of PI3K using wortmannin markedly enhanced LPS and poly(I:C) inducible NF-κB, mitogen-activated protein kinases (MAPKs) and dsRNA activated Ser/Thr kinase PKR activation resulting in heightened IL-12 and TNF-α production. Furthermore, activation of PI3K was found to limit type I IFN-β synthesis and subsequent Stat-1 Tyr 701 phosphorylation in an autocrine/ paracrine fashion induced by LPS and poly(I:C). Accordingly, current experiments are in progress to elucidate the distinct role of PI3K-mediated negative control on NF-κB and ISRE-driven promoters activated by TLR-4/TLR-3 triggering.
Our findings strongly suggest that PI3K represents a critical component of the TLR-4/TLR-3 dependent negative control mechanism that limits the magnitude of the inflammatory responses.

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ROLE OF THE TLR2/MYD88 ACTIVATION PATHWAY IN GROUP B STREPTOCOCCAL INFECTIONS

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Toll-like receptors (TLRs) are involved in pathogen recognition by the innate immune system. Toll-like receptor 2 (TLR2) and the adaptor molecule myeloid differentiation factor 88 (MyD88) were previously shown to mediate *in vitro* cell activation induced by group B streptococcus (GBS). The present study examined the potential *in vivo* roles of TLR2 and MyD88 during infection with GBS. Neonatal TLR2- or MyD88-deficient mice were infected with a full range of GBS doses. When pups were infected locally with a low bacterial dose, none of the TLR2- or MyD88-deficient mice, but all of the wild-type ones, were able to prevent systemic spread of GBS from the initial focus. Bacterial burden was higher in MyD88- than in TLR2-deficient mice, indicating a more profound defect of host defense in the former animals. When a high bacterial dose was used, mutant and wild type mice showed similar high-level bacteremia. Under these conditions, TLR2 or MyD88 deficiency significantly protected mice from lethality, concomitantly with decreased circulating levels of tumor necrosis factor-α and interleukin 6. These data highlight the double role of TLR2 and MyD88 as important host defense factors during initial GBS infection and as mediators of lethality in the presence of overwhelming sepsis.

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ESSENTIAL ROLE FOR MYD88 AND MAL/TIRAP IN RHEUMATOID ARTHRITIS

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The mechanisms that regulate inflammatory and destructive processes in rheumatoid arthritis (RA) remain largely unknown. In this study, we investigated the involvement of MyD88 and Mal/TIRAP, two adaptor molecules involved in toll-like receptor (TLR) signalling, in the regulation of key cytokines and matrix metalloproteinases in synovial tissue from RA patients. Using recombinant adenoviruses to express inhibitory dominant negative forms of these molecules in RA synovial tissue, we found that both MyD88 and Mal/TIRAP are essential for the disease-driven production of IL-6 and IL-8, as well as matrix metalloproteinases (MMP)-1, 2, 3 and 13. Interestingly, MyD88 and Mal/TIRAP are not required for TNFα or IL-1 production in this system, a finding that may reflect the cellular source of these molecules in the RA synovial tissue where the majority of IL-6 and IL-8 comes from fibroblast-like cells whereas the majority of TNFa comes from macrophage-like cells. As Mal/TIRAP is a specific adaptor for TLR but not IL-1R signalling, our results suggest that TLR(s) control inflammatory and destructive processes in RA. The elucidation of this TLR signalling pathway and the identification of the TLR(s) involved may thus provide important targets for therapeutic intervention for this incurable and painful disease.

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T1/ST2 INHIBITS IL-1 AND LPS BUT NOT POLYI: C INDUCED NF-KB ACTIVATION

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A T1/ST2 is a member of the IL-1 receptor family possessing the three characteristic extracellular Ig domains and an intracellular TIR domain. It is an orphan receptor expressed on a variety of cell types including mast cells, naïve T cells and Th2 cells. We have previously shown that T1/ST2 is an active signalling member of this family as it is able to activate the MAP kinases; JNK, p42/p44 and p38. T1/ST2 is not, however, able to activate NF-κB.

Here we show that T1/ST2 acts as an inhibitor of IL-1 and LPS induced NF-κB activation. Using both overexpression of T1/ST2 and a crosslinking monoclonal antibody to T1/ST2, a downregulation of NF-κB activation in cells stimulated with IL-1 and LPS was observed. No inhibition was observed however, of either NF-κB or ISRE in cells stimulated with PolyI:C. Overexpression of T1/ST2 also inhibits NF-κB activation by ILIRAcP, TLR4, Mal, MyD88 but not IRAK or the TLR3 adaptor TICAM-1. A GST fusion of the TIR domain of T1/ST2 is shown to bind to Mal and MyD88 and not TICAM-1 indicating that the observed inhibition of IL-1 and LPS may occur through sequestration of these intermediate signalling molecules. T1/ST2 is, therefore, an inhibitory member of the IL-1 receptor family with respect to NF-κB, consistent with its role in Th2 cell regulation.

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INDUCIBLE IL-10 RECEPTOR DYSFUNCTION – A NEW ROLE FOR TOLL-LIKE RECEPTORS IN THE LUNG

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Despite an immunosuppressive lung environment, alveolar macrophages (AM) retain the capacity to respond to microorganisms. This study evaluated whether constitutive production of interleukin-10 (IL-10) regulates the activity of AM, which can be overcome by stimulation of Toll-like receptors (TLR) on macrophages. IL-10 mRNA and protein were constitutively expressed in normal alveolar epithelium and IL-10 receptors were constitutively expressed on normal AM. Stimulation of AM through TLR2, TLR4 or TLR9 was sufficient to inhibit IL-10R signal transduction, including phosphorylation and nuclear translocation of STAT3 transcription factor. TLR-mediated inhibition of IL-10R function did not alter surface expression of IL-10R, but was dependent on MyD88 adaptor protein expression. Finally, continuous exposure of AM to IL-10 caused sustained expression of the chemokine receptors, CCR1 and CCR5; however, IL-10-mediated expression of these chemokine receptors was rapidly downregulated by the addition of TLR ligands. These findings demonstrate a novel regulatory mechanism that allows AM to overcome the effects of constitutive IL-10 in the lungs in order to respond more effectively to pulmonary infections.

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TOLL-LIKE RECEPTOR 4 POLYMORPHISM NOT ASSOCIATED WITH *H. PYLORI* RELATED INTESTINAL METAPLASIA

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Aim: Toll-like receptor 4 (TLR4), expressed in the gastric mucosal cells, is specifically involved in the initial immune response to gram-negative bacteria. A single nucleotide polymorphism (SNP) has been identified at + 896 of TLR4 (an adenine → guanine substitution). Subjects expressing this SNP have been shown to exhibit reduced expression of TLR4. To date, no studies have investigated the prevalence of this SNP in *Helicobacter pylori (H. pylori*-a Gram-negative bacteria) associated gastritis or intestinal metaplasia (presumptive gastric cancer pre-cursor).

The aim of the present study was to determine the prevalence of the functional single nucleotide polymorphism at position + 896 of the TLR4 gene in an Irish population of *H.pylori* uninfected controls and *H. pylori* positive gastritis and IM groups.

Subjects & Methods: *H. pylori* negative normal controls (n = 96), *H. pylori* positive gastritis (n = 91) and IM (n = 63). A previously published Taqman allelic discrimination assay was used to screen the patients for the polymorphism.

	Genotype			Allele frequency
Patient groups	1,1	1,2	2,2	%
Normal-(H. pylori Neg.)	82	12	2	8.3
Gastritis-(H. pylori Pos.)	77	13	1	7.8
IM-(H. pylori Pos.)	52	9	1	8.9

Results: No deviation from Hardy-Weinberg equilibrium was noted across the total patient cohort. There was no significant association between carriage of the rare allele and gastritis or IM. Adjusting for age and sex did not alter the association.

Conclusion: This study found carriage of the rare TLR4 Asp299Gly polymorphism was not associated with successful *H. pylori* infection or subsequent disease pathogenesis.

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LPS/TLR4 SIGNALING TO IRF3/7 AND NFKB INVOLVES THE TOLL ADAPTERS TRAM AND TRIF

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TRIF-related adapter molecule (TRAM) is the fourth TIR domain containing adapter protein to be described that participates in Toll receptor signaling. TRAM like TRIF, MyD88 and Mal activates NFκB. TRAM also activates IRF3 and IRF7, a property it shares with TRIF. Toll-like receptors (TLR)-3 and -4 activate IRF3, IRF7 and NFκB to induce *IFNA/B* and *RANTES* gene expression independently of the adapter proteins MyD88 and Mal/TIRAP. siRNA, dominant negative and co-immunoprecipitation studies demonstrate that TRIF functions downstream of both the TLR3 (double stranded RNA) and TLR4 (lipopolysaccharide) signaling pathways to IRF3, while the function of TRAM is restricted to the TLR4 signaling pathway. TRAM signaling to IRF3 is inhibited by dominant negative mutants of TRIF. Furthermore, TRAM interacts with TRIF, Mal, TLR4 but not TLR3. TRIF and TRAM likely cooperate to regulate the MyD88-independent pathway downstream of TLR4 during the innate immune response to lipopolysaccharide.

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MYD88-ADAPTER-LIKE (MAL) IS A PHOSPHO-PROTEIN

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Signal transduction by the LPS receptor Toll-like receptor-4 (TLR-4) involves the adapter protein MyD88 adapter-like (Mal, also known as TIRAP). A role for Mal distinct from MyD88 is yet to emerge and evidence so far implicates it only in the activation of NF-kappa B. We have found that when Mal is over-expressed in HEK293 cells, multiple bands appear upon Western blotting. MyD88 on the other hand migrates as a single form. Treatment of lysates containing Mal with calf intestine alkaline phosphatase, results in the disappearance of the slower migrating forms, suggesting that when over-expressed, Mal becomes phosphorylated. Furthermore when lysates from THP-1 cells are incubated with GST-Mal in an *in vitro* kinase assay, Mal becomes phosphorylated. Using an antibody that specifically recognises phospho-tyrosine residues, we determined that Mal is tyrosine phosphorylated. Mal contains six conserved tyrosine residues, all of which are located in the TIR domain. These tyrosine residues were mutated to phenylalanine either individually or in combination. We next preformed western blot analysis of transfected cell extracts to reveal the phosphorylation state of the Mal mutants with respect to the wild-type Mal protein. A severe reduction in the quantity of the slowest migrating form for Y86F, Y106F and Y187F was detected, whereas Y159F, Y195F and Y196F retained the same profile as wild-type Mal. Using an NF-κB dependant luciferase gene, we discovered that when overexpressed the Y86F mutant did not activate NF-κB. In a similar fashion to the MalP125H mutant, MalY86F is capable of blocking the effect of a constitutively active form of TLR4, whereas it had no effect on NF-κB activation following IL-1 stimulation. Collectively our results suggest that the tyrosine residue located at position 86 in box 1 of the TIR domain must undergo phosphorylation in order for Mal to signal.

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STRUCTURAL COMPLEMENTARITY OF TOLL/INTERLEUKIN-1 RECEPTOR DOMAINS

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The Toll/interleukin 1 receptor (TIR) domain is a region found in the cytoplasmic tails of members of the Toll-like receptor/Interleukin-1 receptor superfamily. The domain is essential for signalling and is also found in the adaptor proteins Mal and MyD88 which function to couple activation of the receptor to downstream signalling components. We show that the purified TIR domains of Mal and MyD88 can form stable heterodimers and also that Mal homodimers and oligomers are dissociated in the presence of ATP. To identify structural features which may contribute to the formation of signalling complexes we produced models of the TIR domains from human Toll-like receptor 4 (TLR4), Mal and MyD88. Docking studies suggest that Mal and MyD88 bind to different regions in TLRs 2 and 4, a finding consistent with a cooperative role of the two adaptors in signalling. Mal and MyD88 are predicted to interact at a third non-overlapping site suggesting that the receptor and adaptors may form heterotetrameric complexes. The theoretical model of the interactions is supported by experimental data from GST-pulldowns and co-immunoprecipitations. Neither theoretical nor experimental data suggest a role for the conserved proline in the BB-loop in the association of TLR4, Mal and MyD88.

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THE NON-CANONICAL IκB KINASES, IKKE AND TBK1, ARE ESSENTIAL COMPONENTS OF THE VIRAL AND TLR SIGNALING PATHWAYS LEADING TO IRF-3 ACTIVATION

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Type I interferon (IFN) and chemokines such as RANTES play a central role in coordinating the innate immune response to viral and microbial infection. The transcription factor interferon regulatory factor 3 (IRF-3) is activated by virus infection or engagement of Toll-Like Receptors (TLRs) by dsRNA or LPS. Upon stimulation, IRF-3 is phosphorylated and translocates to the nucleus where it activates the expression of type I IFN, RANTES and interferon stimulated genes. The IκB kinase homologues, IκB kinase epsilon (IKKe) and TANK-binding kinase-1 (TBK1) have been previously implicated in activation of the transcription factor NF-κB. Here we show that IKKe and TBK1 are essential components of the virus-dependent as well as TLR dependent activation of IRF-3. In addition, the TLR adapter molecule, TRIF, lies upstream of IKKe and TBK1 in the TLR-dependent signaling pathway leading to the induction of IRF-3. We conclude that IKKe and TBK1 are pivotal regulators of both the IRF-3 and NFκB signaling pathways in the innate immune response.

VIRAL CYTOKINE MECHANISMS

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HERPES SIMPLEX VIRUS TYPE 1 SUPPRESSES PROINFLAMMATORY CYTOKINE PRODUCTION *IN VITRO* AND *IN VIVO* THROUGH THE VIRAL IMMEDIATE-EARLY GENES ICP4 AND ICP27

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Immune evasion represents an important mechanism of pathogenesis. Many viruses are in possession of mechanisms to counteract the antiviral response raised by the infected host. Here we show that a herpes simplex virus type 1 (HSV-1) mutant lacking functional viral protein (VP)16 – a tegument protein promoting expression of viral gene expression – induces significantly higher levels of proinflammatory cytokines than wild-type HSV-1. This was observed in several cell lines and primary murine macrophages as well as in peritoneal cells harvested from mice infected *in vivo*. The elevated ability to stimulate cytokine expression in the absence of VP16 was not mediated directly by VP16, but was dependent on the viral immediate-early genes infected cell protein (ICP)4 and ICP27, which are expressed in a VP16-dependent manner during primary HSV infection. The virus targets cellular mechanisms independent of the dsRNA-activated protein kinase R (PKR), since the virus mutants remained stronger inducers of cytokines in cells stably expressing a PKR mutant lacking the first RNA-binding domain. Finally, the mechanism through which HSV-1 down-modulates cytokine expression is exerted at the post-transcriptional level. Thus, HSV is able to suppress expression of proinflammatory cytokines, and may thus impede the antiviral host response to the infection.

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INHIBITION OF IL-1-INDUCED INFLAMMATION IN BRONCHIAL EPITHELIAL CELLS BY *VACCINIA* VIRUS PROTEINS A46R AND A52R

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Cystic Fibrosis is characterized in the lungs by neutrophil-dominated inflammation mediated by several pro-inflammatory stimuli, including *Pseudomonas* lipopolysaccharide (LPS), tumour necrosis factor-((TNF(), neutrophil elastase (NE), and interleukin-1 (IL-1). Previous work from our laboratory has elucidated the intracellular mechanisms by which NE up-regulates inflammatory gene expression in bronchial epithelial cells. In this study we examined the effects of IL-1 stimulation on bronchial epithelial cells and investigated the intracellular pathways involved in IL-1 signal transduction. RT-PCR analysis demonstrated that IL-1 receptor type 1 (IL-1R1) and IL-1 receptor accessory protein (IL-1RAcP), components of the IL-1 receptor complex are expressed in 16HBE14o- cells. Stimulation of 16HBE14o- cells with IL-1 dose-dependently induced IL-8 protein production as shown by ELISA. Western blot analysis implicated IRAK-1, but not IRAK-2 or IRAK-4, degradation in the regulation of these events. Furthermore, we observed that co-transfection with cDNAs expressing the *Vaccinia* viral proteins, A46R and A52R, significantly abrogated IL-1-induced NF(B up-regulation. These results show that IL-1 induces IL-8 production in bronchial epithelial cells through an IRAK signalling pathway, and that inflammatory gene expression can be inhibited by the *Vaccinia* virus proteins, A46R and A52R. These findings identify possible therapeutic targets for the inflammatory manifestations of cystic fibrosis.

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INTRACELLULAR MAPK SIGNALLING PATHWAYSARE INVOLVED IN SIVAGM3-INDUCED UPREGULATION OF IL-2 GENE EXPRESSION

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Immunodeficiency virus infection is characterized by a broad immunodysregulation with an altered pattern of secreted cytokines. Interleukin-2 (IL-2), the major T cell growth cytokine, was shown to be highly expressed in the lymph nodes of HIV-1-infected individuals and is supposed to have a functional influence on replication and pathogenesis of human/simian immunodeficiency viruses (HIV/SIV). To study the immunomodulation induced by apathogenic SIVagm3 we performed IL-2-specific intracellular immunohistochemical staining of the lymph nodes of African green monkeys (agm) chronically infected with SIVagm3 and observed pronounced IL-2 expression. Using the infectiousmolecular clone of SIVagm3 in transient transfection experiments we show that SIVagm3 induced 38-fold increased transcriptional activation of the IL-2 promoter. Inhibition of mitogen-activated protein kinase (MAPK) signalling pathways by treating T cells with inhibitors of MEK, JNK or p38 abolished this activation in a dose-dependent manner, whereas the classical IL-2 inhibitor cyclosporin A (CyA) had no effect. Analysing critical transcription factor binding sites located on the IL-2 promoter we found that SIVagm3 did mainly promote transcriptional activation of the CD28/AP-1 responsive element. A strong increase in IL-2 promoter-dependent transcription was also detected when the SIVagm3 transactivator protein (Tat), which is able to activate the CD28/AP-1 responsive element, was overexpressed in activated T cells. These results show that apathogenic SIVagm3 modulates IL-2 expression in the target cells via intracellular MAPK signal transduction. Transcriptional activation of the IL-2 promoter is at least partly mediated by the viral Tat protein that acts via the CD28/AP-1 responsive element.

LATE ABSTRACTS

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HALOFUGINONE - A CYTOKINE PRODUCTION ENHANCER

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Halofuginone (HF), a low molecular weight quinazolinone plant alkaloid, known to inhibit collagen type I and matrix metalloproteinase 2 (MMP-2) gene expression and synthesis, was previously shown to inhibit angiogenesis progression, neovascularization and tumor growth. This study aimed to evaluate the effect of Halofuginone (HF) on inflammatory (IL-1ß, IL-6, IL-8, TNFα) and anti-inflammatory (IL-10, TGFß) cytokines production in vitro, by normal human blood monocytes, from 20 healthy volunteers.

HF (50, 100 nM) increased significantly the production of IL-1ß (450%) - similar to LPS, however, the increase in IL-6 (300-500%) and in TNFα (40-60%) was lower than the effect of LPS on unstimulated monocytes.

HF (100 nM) decreased slightly IL-8 production by unstimulated monocytes, unlike LPS - which stimulated significantly IL-8 production.

When the effect of HF (50, 100 nM) was tested on LPS stimulated monocytes, it induced an additional increase in the production of IL-1ß, IL-6 and a decrease in IL-8 and TNFα by the LPS stimulated monocytes.

Halofuginone (50, 100 nM) decreased significantly (~ 50%) the production of TGFß in vitro by non-activated normal human blood monocytes and had no effect on LPS activated monocytes. IL-10 production was increased (~ 50%) by HF (50 nM).

Taken together, HP is a strong inducer of inflammatory cytokines and some of these effects may explain his described activities in the inhibition of angiogenesis and tumor growth.

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CELL-SPECIFIC DIFFERENCES IN NUCLEAR TRANSCRIPTION FACTORS RESPONSIBLE FOR IL-1b INDUCTION BY S100B

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Both the astrocyte-derived cytokine S100B and the proinflammatory cytokine interleukin-1 (IL-1) are elevated in Alzheimer brain, and overexpression of each is implicated in β-amyloid plaque formation and neurofibrillary pathology. The parallel elevation of S100B and IL-1 suggests that a link between these cytokines orchestrates glial-glial and glial-neuronal interactions that give rise to neuronal dysfunction. To address relevant mechanisms, we examined the gene regulatory events through which S100B induces IL-1 expression in microglia and neurons. Treatment of microglia with S100B activated NFκB within 2 h, followed by an increase in IL-1 expression. The IL-1 induction was inhibited by loading the cells with double-stranded oligonucleotides containing NFκB binding sites to serve as «decoy» DNA and thereby reduce available NFκB. Conversely, S100B induced IL-1 expression in cortical neurons without NFκB activation. Instead, a transient elevation of Sp1-related binding activity preceded the increase in IL-1 expression in neurons. This was suppressed with a Sp1 «decoy» strategy. Our results suggest that the overexpression of S100B and IL-1 in Alzheimer brain is mediated through cell-specific transduction pathways, further linking chronic glial activation and subsequent neuroinflammation and neuronal dysfunction.

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COMBINED STIMULATION WITH IL-4 AND IL-10 INDUCES MAST CELL APOPTOSIS VIA A P53-DEPENDENT MECHANISM

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IL-4 and IL-10 are central regulators of Th2-mediated immunity. Because mast cells are one of the effectors of the Th2 response, we assessed the effects of IL-4 and IL-10 on mast cell function and survival. We have previously shown that IL-4 and IL-10 inhibit expression and function of FcRI and c-Kit. In this study we demonstrate that combined stimulation with IL-4 and IL-10 induces apoptosis in murine mast cells. Cells stimulated with IL-4 and IL-10 show decreased expression of bcl-2 and bcl-xl and loss of mitochondrial membrane potential. Mast cells were derived from several gene-deficient or transgenic mice. Studies with these cells indicated that loss of Stat6, p53, or Bax, or overexpression of Bcl-2 prevent cytokine-induced mast cell apoptosis. These data indicate that the Th2 cyokines IL-4 and IL-10 participate in regulating mast cell function and survival, comprising part of what we believe is a cytokine-mediated mast cell homeostatic network.

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SCHLAFEN-1 is A NOVEL INHIBITOR OF THE CELL CYCLE

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The schlafen (Slfn) family of genes have been previously described to be differentially regulated during thymocyte maturation and are preferentially expressed in lymphoid tissues. This family of genes includes a viral member acquired by poxviruses from host cells. Slfn-1, the prototype mammalian family member has been shown to cause a growth arrest in NIH3T3 cells prior to the G1/S transition when induced in a tetracycline inducible system. We have been investigating the mechanism of Slfn-1-mediated growth arrest by analysing mitogenic signalling in this system.

When induced to express Slfn-1, these cells exhibit profound defects in PDGF signalling to Akt, Ras, Raf-1, MEK1/2 and ERK1/2. Other signalling pathways, such as those activated by Interleukin-1 (IL-1), Tumour Necrosis Factor (TNF), Lipopolysaccharide (LPS), Epidermal Growth Factor (EGF) and Phorbol myristate (PMA) are unaffected. We have linked these effects to a decrease in PDGF receptor levels, a phenomenon reversible on suppression of Slfn-1 expression. Also, Slfn-1 induced cells are unable to induce D-type cyclins on stimulation with serum and exhibit severely reduced levels of other cell cycle regulators compared to non-induced cells. These data support the identity of Slfn-1 as a novel inhibitor of the cell cycle.

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CYTOKINE POLYMORPHISMS: ALLELE, GENOTYPE AND HAPLOTYPE FREQUENCIES IN THE IRISH POPULATION

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Background: Genetic susceptibility to a range of diseases has been linked to various cytokine SNPs. Different promoter region SNPs have been reported to give rise to variable levels of expression in cytokines such as TNFα and IL-10. IL-6, TNFα and IL-10 SNPs have been associated with increased risk of graft versus host disease and renal graft rejection.
Objectives: To establish cytokine SNP allelic and haplotypic frequencies in a healthy Irish population cohort. This data will be useful to researchers carrying out disease association and genetic susceptibility studies where cytokine activity may be involved.
Materials and methods: The primers used for cytokine genotyping allowed for the direct typing of SNP haplotypes in individuals tested. 200 healthy Irish subjects who had already been HLA typed were typed for 22 cytokine SNPs. Polymorphisms in the promoter regions of IL-1α, IL-1β, IL-1R, IL-1RA, IL-12, INFγ, TNFα, IL-2, IL-4, IL-6, IL-10 and the translated regions of IL-4RA and TGFβ were examined.
Results: Allelic, genotypic and haplotypic frequencies were obtained. All of the 22 SNPs tested were in Hardy-Wienberg equilibrium. IL-1α -889T and IL-1β+ 3962T were found to be in linkage disequilibrium. TNFα -238A and HLA-B*57 were in linkage disequilibrium as were TNFα –308A and HLA-B*08.

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ANTI-INFLAMMATORY EFFECTS OF CANNABINOID RECEPTOR LIGANDS IN GLIAL CELLS

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Cannabis (marijuana) demonstrates therapeutic effects in the treatment of inflammatory-based conditions such as multiple sclerosis. Whilst the psychoactive components of marijuana are known to act via the CB1 and CB2 cannabinoid receptors the mechanism(s) underlying its therapeutic effects is ill-defined. This study shows that the endogenous cannabinoid receptor ligand anandamide and synthetic cannabinoids reduce the capacity of IL-1 to induce the expression of adhesion molecules and chemokines in astrocytes, likely key processes in the generation of multiple sclerosis. However the use of selective CB1 and CB2 agonists and antagonists suggest that the inhibitory effects of anandamide are not exclusively mediated via these receptors. This is confirmed by the lack of expression of the receptors in the glial cells as determined by RT-PCR. This work also proposes a mechanism for the anti-inflammatory effects of cannabinoids and demonstrate that such a mechanism is independent of the CB1 and CB2 receptors.
This work was funded by Enterprise Ireland.

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A DISTINCT CYTOKINE RESPONSE IS OBSERVED IN HCV-INDUCED HEPATIC CIRRHOSIS

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The hepatic cytokine network is active even in healthy liver. The normal hepatic cytokine balance is critical for control of infection as well as maintaining normal hepatic architecture and function. Liver contains distinct populations of immune cells, as well as other parenchymal and non-parenchymal cells, which produce cytokines in response to infection and stress. The cytokine imbalance resulting from chronic inflammation in the liver promotes the development of fibrosis and cirrhosis. The aim of this study was to compare the changes in the hepatic cytokine environment associated with cirrhosis and, to determine if specific changes occur in HCV-related cirrhosis. We obtained normal liver biopsies from donor organs (n = 12) prior to liver transplantation. HCV-infected (n = 11) and cirrhotic control liver (ALD n = 10; PBC n = 10) was obtained at time of liver transplantation for end-stage liver disease. Liver was snap-frozen and powdered in liquid nitrogen, soluble protein was extracted and cytokine levels were measured by ELISA. IL-18, IFN-□ and IL-10 were significantly increased in all cirrhotic tissues ($p<0.05$). However, the magnitude of the changes observed for IFN-□ (x15.6) and IL-10 (x3) in HCV-associated cirrhosis differed significantly from both ALD (IFN-□ x3.33; IL-10 x8.52, $P<0.05$) and PBC (IFN-g x2.66; IL-10 x9.6, $P<0.05$). Surprisingly, the levels of IL-12 and IL-2 were unchanged in HCV-cirrhosis although significant and equivalent increases were observed for these cytokines in cirrhotic controls. An almost 5-fold increase was observed in IL-15 levels in HCV-cirrhosis ($p<0.05$) whereas no change occurred in ALD/PBC. In conclusion, a distinct cytokine response was observed in hepatic cirrhosis caused by HCV infection when compared with autoimmune- and alcohol-induced cirrhosis. These findings have important implications for our understanding of the mechanisms involved in the generation of cirrhosis and viral persistence in chronic HCV infection.
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IκBβ: THE KEY TO SUSTAINED ACTIVATION OF NFκB IN GLIAL CELLS

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NFκB is an inducible transcription factor, which regulates the expression of a variety of cellular genes, including many of those involved in immune and inflammatory responses. It is activated by pro-inflammatory cytokines such as IL-1 and TNF. In unstimulated cells, NFκB is sequestered in the cytoplasm by the inhibitor of kappa B (IκB) proteins, the most common members of this family being IκBα and IκBβ. Most pathways causing NFκB activation result in the stimulation of the IκB kinase (IKK) complex, which is followed by phosphorylation of the IκB protein at specific serine residues. This triggers ubiquitination and degradation of the IκB proteins. We have shown that IL-1 causes persistent activation of NFκB in astrocytes. IκBα is rapidly but transiently degraded. NFκB remains active despite the return of IκBα to control levels. However IκBβ is not resynthesized, remaining absent throughout the persistent activation of NFκB. Proteasome inhibition during IL-1-mediated NFκB induction lead to the reappearance of a protein of similar electrophoretic mobility to IκBβ and caused decreased NFκB activity. This suggests that immediate degradation of newly-synthesized IκBβ, mediated by the proteasome, could underlie the failure of astrocytes to re-accumulate the protein despite the presence of the IκBβ transcript, and contribute to the persistent activation of NFκB.
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NEGATIVE REGULATORS OF CYTOKINE SIGNALLING

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Cytokines are an integral component of the adaptive and innate immune responses. The signalling pathways triggered by the engagement of cytokines with their specific cell surface receptors have been extensively studied and have provided a profound understanding of the intracellular machinery that translates exposure of cells to cytokine to a coordinated biological response. It has also become clear that cells have evolved sophisticated mechanisms to prevent excessive responses to cytokines. The suppressors of cytokine signalling (SOCS) are a family of cytoplasmic proteins that complete a negative feedback loop to attenuate signal transduction from the hematopoietin class of cytokine receptors. SOCS proteins inhibit components of the cytokine signalling cascade via direct binding or by preventing access to the signalling complex. The SOCS proteins also appear to target signal transducers for proteasomal destruction. Analysis of genetically modified mice in which SOCS proteins are overexpressed or deleted have established that this family of negative regulators has indispensable roles in regulating cytokine responses in cells of the immune system as well as other tissues. Emerging evidence also suggests that disruption of SOCS expression or activity is associated with several immune and inflammatory diseases, raising the prospect that manipulation of SOCS activity may provide a novel future therapeutic strategy in the management of immunological disorders.

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THE ROLE OF MICROENVIRONMENTAL IL-1 in TUMOUR DEVELOPMENT

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We have assessed the role of host-derived IL-1 on the development of chemically induced tumors using knockout (KO) mice which lack specific IL-1 genes, i.e., IL-1α, IL-1β and IL-1α/β (double KO). These mice were injected with 3- methyleholanthrene (3-MC). It was shown that IL-1 β and IL-1α/β KO mice are less susceptible to the induction of fibrosarcomas as compared to control or IL-1α KO mice. 3-MC induced tumour cells lines obtained from control BALB/c mice were shown to grow rapidly in IL-1Ra KO mice, indicating the role of host-derived IL-1 in tumor invasiveness.
In 3-MC-treated IL-1α KO mice immunogenic tumor cells had arisen (these cells do not grow in intact control mice, but develop into tumors in sublethal-irradiated mice). These cells all express ICAM- 1, while no ICAM-1 expression was observed in WT mice. These adhesion molecules serve as cell-associated co-stimulatory molecules which induce high affinity biding of the malignant cells to immune effector cells, resulting in better killing and the eradication of the malignant cells. Altogether, our results clearly indicate that different IL- 1 molecules have distinct functions in various phases of the malignant process, carcinogenesis, tumour cell invasiveness and selection of tumor cell variants according to their immunogenicity.

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EXTRACELLULAR HIV-1 TAT INDUCES T CELLS TO PROLIFERATE AND PRODUCE IFN-γ DIRECTING A TH1 IMMUNE RESPONSE

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T cells undergo a Th1 or Th2 kind of immune response upon activation. Accordingly, they secrete distinct classes of cytokines or chemokines that aid in T cell-T cell interaction or T cell-B cell interaction. A Th1 response includes the production of IFN-γ, IL-2, etc. A Th 2 response involves the production of IL-4, IL-6 etc. Different stimuli induce such signals and mitogens like PMA, ConA secrete both kinds of cytokines. Under some conditions costimulatory molecules along with the TCR-induced signal are required for activation. Recently, by microarray analysis, it has been shown that overexpression of soluble Tat induces IFN-γ responsive genes. We find that extracellular HIV-Tat also upregulates IFN-gamma in T cells, directing a Th1 kind of an immune response. IFN-γ production involves signal transducing proteins like those of the JAK-STAT pathway and various transcription factors especially STAT1. Although there have been the involvement of PKC and integrins in signalling, the entire pathway or the exact molecules acting, have not been identified. We hypothesise that the involvement of this cytokine and its correlation with Tat, could be of potential use in HIV pathogenesis and its regulation.

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INDUCTION OF ANTIGEN-SPECIFIC REGULATORY T CELLS *IN VIVO* USING ADJUVANTS THAT MODULATE DENDRITIC CELL ACTIVATION

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Cholera toxin (CT) is a potent mucosal adjuvant, enhancing Th2 or mixed Th1/Th2 type responses to co-administered antigens. We found that CT also promotes the generation of type 1 regulatory T (Tr1) cells. Parenteral immunization of mice with antigen and CT induced T cells that secreted high levels of IL-4 and IL-10 and lower levels of IL-5 and IFN-γ. Antigen-specific $CD4^+$ T cell lines and clones generated from these mice had cytokine profiles characteristic of Th2 or Tr1 cells and could suppress IFN-γ production by Th1 cells. Furthermore, adoptive transfer of bone marrow derived dendritic cells (DC) incubated with antigen and CT induced IL-10-secreting T cells. We have previously reported that IL-10 may act as a differentiation factor for Tr1 cells. Here we found that CT synergized with LPS to induce IL-10 production by immature DC. CT also enhanced expression of CD80, CD86 and CD134 on DC but inhibited LPS-driven induction of CD40 and ICAM-1 expression and production of inflammatory chemokines and cytokines. The findings suggest that CT induces maturation of DC, but by inducing IL-10 and inhibiting IL-12 and selectively affecting surface marker expression suppresses the generation of Th1 cells and promotes the induction of Tr cells.

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INVOLVEMENT OF GRAFT-DERIVED IL-15 IN ACUTE PANCREATIC ISLET GRAFT REJECTION IN MICE

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IL-15-mRNA levels are elevated in acutely rejected renal transplants in patients and in islet grafts in mice, suggesting a role for IL-15 in the process of acute graft rejection. We previously reported that human tubular epithelial cells produce IL-15 that promotes lymphocyte proliferation in a mixed culture. In the present study, we use murine-IL-15-antisense to inhibit islet IL-15 production in a mouse model of acute islet graft rejection. 500 islets were purified from B10.BR mice ($H\text{-}2^k$), incubated for 1 hour with either antisense (0.1 μM) (n = 9), a control scrambled oligodeoxynucleotide (n = 8) or medium (n = 5), and transplanted under the renal capsule of minor-MHC disparate CBA mice ($H\text{-}2^k$), rendered diabetic by streptozotocin. Similarly, islets were purified from C57BL mice ($H\text{-}2^b$). Blood glucose >300 mg/dL designated graft rejection. In the minor-MHC disparate pairing, we observed a median graft survival time of 25 and 17 days in the untreated and scramble treated groups, respectively. However, in the antisense treated group, eight out of nine grafts exhibited indefinite graft survival (>120 days, $p<0.05$). Antisense treatment did not affect graft survival of major-MHC pairing. These findings suggest that graft-derived IL-15 is involved in acute islet graft rejection, and support the opinion that the graft actively regulates the process of cellular immune rejection. To determine the effect of IL-15 inhibition on graft survival between major-MHC disparate mice, a treatment protocol that combines IL-15-inhibition and sub-therapeutic doses of immunosuppressant drugs should be attempted.

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CONSTITUTIVE STAT3 ACTIVATION IN CROHN'S DISEASE: ROLE IN THE IMMUNOPATHOGENESIS AND NEW PROSPECTIVE FOR IMMUNOTHERAPY

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Via cytoplasmic signal transduction pathways, cytokines induce a variety of biological responses and modulate the outcome of inflammatory diseases and malignancies. Crohn's disease (CD) is a chronic inflammatory bowel condition, affecting young adults and, indeed, is a severe medical- and socio-economic strain, both to the individual patient and the community. Although the etiology is unknown, several findings suggest that perturbation of the intestinal cytokine homeostasis and exacerbated T cell response towards normally occurring gut proteins play a central pathogenic role in CD. Here, we study intestinal T cells from CD and healthy volunteers. We show that Stat3 is constitutively activated by Jak3 and that the Stat3 regulated protein, SOCS3, is also constitutively expressed in Crohn's patients but not in healthy volunteers. Furthermore, constitutive Stat3 activation is found also in peripheral T cells from CD patients but not in the controls, indicating that disturbances in Stat-activation are a generalized phenotype of T cells from CD patients. This aberrant activation, so far noted only in malignant cells, suggest a possible pathogenetic link between cancer and autoimmunity and establish new critical approaches for better understanding the immunopathogenesis of CD and new prospective in fighting chronic inflammatory bowel disease and possibly other autoimmune conditions.

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MAL/TIRAP UNDERGOES TLR-2/TLR-4-MEDIATED DEGRADATION AND INTERACTS WITH TRAF6 TO INDUCE NF-κB TRANSCRIPTIONAL ACTIVATION

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Mal/TIRAP was described as a cytosolic MyD88-like adapter involved in Toll-like receptor [TLR]-4-mediated activation of NF-κB. However, studies of $Mal^{-/-}$ mice demonstrated that while the 'MyD88-independent pathway' was intact, LPS signalling was delayed, similar to $MyD88^{-/-}$ mice. Suprisingly, Mal was found to be critical for TLR2-mediated responses. Importantly, these studies failed to reveal a specific function for Mal in TLR-mediated activation of NF-κB and immune host response.
In this study, we demonstrate that Mal undergoes TLR2- and TLR4-mediated degradation via a putative PEST domain. The proteasome inhibitor MG132 can abolish this degradation. Furthermore, Mal was found to interact with TRAF6, presumably via a putative TRAF6-binding motif found in the TIR domain of Mal.
A functional role for this interaction in Mal-dependent transcriptional activity was suggested by the inability of MalE290A, containing a point mutation within this putative TRAF6-binding domain, to drive NF-κB-dependent reporter gene expression. Furthermore, expression of MalE290A inhibited TLR-mediated activation of NF-κB.
Therefore, the combination of ligand induced degradation of Mal, and the functionality of Mal/TRAF6 interaction in NF-κB activation and gene expression, may suggest a role for Mal in TLR2- and TLR4-mediated tolerance.

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A NEW, FLEXIBLE ASSAY FORMAT FOR QUANTIFICATION OF MULTIPLE PROTEINS IN <20 μL SERUM

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In drug discovery small animal models are used to study the effect of drug candidates on the concentration of representative proteins in serum. Sample availability, typically 20 μl per animal, severely limits the possibility of assaying more proteins in a shorter time. Conventional technologies are poorly designed for handling small volumes.
To overcome these difficulties a flexible assay format for protein quantification has been miniaturized and integrated into a CD microlaboratory.
A CD contains microstructures in which samples are assayed in parallel as the CD spins. Each microstructure contains a column (10-15 nl) prepacked with streptavidin-coated beads. Biotinylated capturing molecules, selected for the proteins of interest, are bound to the beads, creating protein-specific columns. Samples (200 nl) pass through the columns, followed by complementary, fluorescently-labelled detecting molecules. Specifically-bound proteins are measured on-line by laser induced fluorescence. Parallel processing speeds up handling and improves reproducibility. Picomolar concentrations can be quantified at precision levels ~ 5-10% CV.
Miniaturization reduces volume requirements for sample and reagent. A protein is quantified using <1 μl of sample. Potentially >10 different proteins could be quantified simultaneously from a 20 μl sample, increasing information content and providing results within hours.

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CRUCIAL ROLES OF T CELL-MEDIATED IMMUNITY FOR CHRONIC AORTITIS IN IL-1 RECEPTOR ANTAGONIST DEFICIENT MICE

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IL-1 receptor antagonist-deficient ($IL\text{-}1Ra^{-/-}$) mice spontaneously develop inflammatory diseases such as chronic arthritis and coronary arteritis. Here, we demonstrate $IL\text{-}1Ra^{-/-}$ mice on the BALB/c background develop arterial inflammation of the aorta with massive infiltration of monocytes, lymphocytes, and infrequently chondrocyte-like cells. We observed left ventricular hypertrophy and mild aortic stenosis in these mice. In rare cases, we also found that $IL\text{-}1Ra^{-/-}$ mice developed myocarditis in the subepidermal pericardium. $IL\text{-}1Ra^{-/-}$ mice develop arterial inflammation independently of joint inflammation, and the onset of aortitis was earlier than arthritis. These findings suggest the difference of the developmental mechanism between aortitis and arthritis in this mouse model. Bone marrow cell- and T cell-transplantation suggest involvement of these cells in the development of aortitis. Furthermore, TNFα-deficiency completely suppressed development of aortitis in $IL\text{-}1Ra^{-/-}$ mice, while IL-6-deficiency failed to protect. Thus, our results demonstrate critical roles of cells of the immune system and specific proinflammatory cytokines including IL-1 and TNFα in the development of aortitis.

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INHIBITION OF HISTONE DEACETYLASES PROTECTS AGAINST ISCHEMIA-INDUCED ACUTE RENAL FAILURE (ARF) IN MICE

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We have previously demonstrated that histone deacetylase inhibitor, suberoylanilide hydroxamic acid (SAHA) reduces the production of proinflammatory cytokines stimulated by LPS, Staphylococci or cytokines themselves (1). We also reported that in ischemic ARF, IL-18 accounts for the deleterious effects of renal damage (2). In the present study, we determined whether SAHA protects against ARF in mice. SAHA (60 mg/kg) or vehicle was administered ip 40 min prior to clamping of the renal pedicles. After 24 hours, animals were sacrificed. Serum creatinine, an indicator of kidney function, was measured. In SAHA ($n = 8$) and vehicle ($n = 5$) treated groups, serum creatinine (mg/dL) was 0.65 and 2.44 ($P < 0.01$), respectively. In sham operated group, creatinine was 0.233. Protective effect of SAHA was also observed morphologically by acute tubular necrosis score. Kidney tissue levels of IL-18 were 2.7 and 5.2 pg/mL ($P< 0.01$) in SAHA and vehicle treated groups, respectively. The role of IL-18 in pathogenesis of ischemic ARF was also supported by experiments in IL-18 binding protein (IL-18BP) overexpressing transgenic mice. The animals overproducing IL-18BP were protected against ischemic ARF compared to littermate controls. In conclusion, SAHA protects against ischemic ARF in mice, likely by reducing of IL-18 activity.

1 Leoni F, et al. Proc Natl Acad Sci USA 2002;99:2995-3000.
2 Melnikov VY. et al. J Clin Invest. 2001;107:1145-52.

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CYTOKINES SYNTHESIS CHARACTERISTICS IN GASTRIC CANCER PATIENTS

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It is known, that cytokines carry out important regulatory role and take part in the organism protection against malignant growth. As the knowledge on immune response formation in cancer is still rather scarce, it was of interest to investigate a condition of IFN system and cytokines in patients with gastric cancer.
Altogether surveyed were 40 healthy volunteers and 20 patients with gastric cancer (IV stage) following palliative gastrectomy and complex chemotherapy. In all of them parameters of IFN status were studied using biological test, while PCR method was used to study the cytokine profile.
In comparison with healthy volunteers the rate of revealed mRNA for IFN-α and IL-8 was increased in patients with gastric cancer at about 90%, while mRNA encoding IL-1 β, IL-6, IL-10, and IL-18 in the patient group grew about 3-4-fold. Meanwhile the number of the patients with revealed mRNA for IFN-γ, IL-12, and TNF-α was decreased accordingly about 6-fold, 4.5-fold, and 4-fold in comparison with healthy donors. Only mRNA for IL-2 and IL-4 were defined in the patients with gastric cancer in 5% of cases, as well as in volunteers.
Our data testify about inhibition of Th1 cells-produced IFN-γ gene expression, as well as IL-12, which activates IFN-γ production by natural killer cells. Meanwhile the amplification of IL-10 gene expression was noted, IL-10 being the inhibitor of IFN-γ and IL-12 synthesis. Probably, this is the cause for infringement of IFN-γ synthesis in the gastric cancer patients registered by a biological method (IFN status).
In most patients (80%) we were not able to reveal mRNA for TNF-α, the cytokine having selective cytotoxicity for tumour cells. I.e. it is possible to assume, that the mechanism of TNF-α synthesis is broken already at a transcription level.
We also found the activation of expression of genes encoding proinflammatory cytokines IL-1β and IL-6, as well as IFN-α and IL-8. To the contrary, the decrease of IFN-α production at the surveyed patients was shown. I.e. it is possible to assume infringement of IFN-α synthesis mechanisms at a level of translation, and/or production.
Altogether our data testify to nfringement of mechanisms of cellular and humoral immunity regulation, as well as synthesis of antiinflammatory and proinflammatory cytokines in patients with gastric cancer following operation and chemotherapy.

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WITHDRAWN

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ANALYSIS OF THE EXPRESSION OF INFLAMMATORY CYTOKINES OVER TIME IN IL-10 KNOCK-OUT MICE

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Background: Inflammatory bowel disease is a chronic immunoinflammatory disorder of the gastrointestinal tract. The IL-KO mouse has served as a model for investigating the pathophysiology of this disorder. It is believed that disruption of the homeostatic cytokine balance underpins much of the immunopathology of this condition.
Aim: The aim of this study was to demonstrate by semi-quantitative RT-PCR with densitometry the expression over time of proinflammatory cytokines in the IL-10 KO model. RNA isolated from colonic epithelium of 17 IL-10KO mice at 4, 16 and 29 week periods was reverse transcribed. Normalised cDNA samples were used as template for subsequent semi-quantitative investigation of IL-1beta, TNFalpha and IFNgamma expression over time. In addition, the results were confirmed by densitometric analysis.
Results: Our results verify the increasing expression of IL-1beta, TNFalpha and IFNgamma at the RNA level as IL-10KO mice age and as disease progresses. Histochemical staining of tissue sections showed histological correlation of inflammation with the cytokine changes over the same time intervals.
Conclusion: IL-1beta, TNFalpha and IFNgamma in IL-10 KO mice increases over a 7 month period which parallels the increase in inflammatory activity and predicted disease state.

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4-1BB ENHANCES CD8$^+$ T CELL EXPANSION BY REGULATING CELL CYCLE PROGRESSION THROUGH CHANGES IN EXPRESSION OF CYCLINS D2 AND E AND CYCLIN-DEPENDENT KINASE (CDK) INHIBITOR p27^{kip1}

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CLONING AND FUNCTIONAL CHARACTERISATION OF HUMAN ECSIT

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Toll Like Receptors and IL-1 signal through largely identical intracellular signalling cascades ultimately activating transcription factor activation such as NFκB and AP-1. The mouse protein ECSIT (Evolutionarily Conserved Intermediate In Toll pathways) has previously been shown to activate NFκB and AP-1 via processing of MEKK-1. This protein is highly conserved, particularly amongst mammals. A dominant negative form ($mECSIT_{261-435}$) had also been shown to block this action. We have cloned the human form of ECSIT (hECSIT). We show that overexpression of hECSIT is sufficient to activate NFκB and AP-1 and induce the chemokine IL-8. Interestingly, we also identify a key arginine in hECSIT. The mutation of this arginine residue abolishes all of the above effects. Such mutation most likely leads to misfolding of hECSIT since the mutant is expressed at comparable levels to wild type hECSIT. Finally we present mutant data relating to the structural requirements of ECSIT with respect to activation of MAP kinase pathways.
This work was funded by the European Biotechnology 5th Framework programme (Contract Nos. QLG1-CT-1999-00549 and QLK3-CT-2000-00270).

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AN ANTI-MURINE IL-22 MONOCLONAL ANTIBODY DECREASES DISEASE SEVERITY IN A MURINE MODEL OF COLLAGEN INDUCED ARTHRITIS

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Interleukin 22 (IL-22) is a cytokine whose function is not well defined. The delivery of an adenovirus encoding murine IL-22 cDNA into C57BL/6J mice altered cellular and serum chemistry changes, induction of acute phase genes and proteins, suggesting that IL-22 induces a systemic acute phase response. *In situ* hybridization of paws from animals with collagen induced arthritis (CIA) showed that both IL-22 and IL-22R mRNAs are increased in diseased joints. A neutralizing rat anti-mouse IL-22 antibody was administered to DBA/1 mice before and after the onset of disease. The treatment of mice with the IL-22 antibody, compared to the control-treated mice, caused both a decrease in clinical scores and joint pathology, as assessed by histology. No significant changes were observed in anti-collagen II antibody levels in treated animals. The acute phase protein SAA was slightly decreased in animals treated with anti-IL-22 antibody. These results suggest that IL-22 may contribute to the inflammatory process in arthritic diseases.

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IRF-3, -5, and -7 are TARGETS OF TLR-7/8 SIGNALING

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Type I interferon (IFN) and chemokines such as RANTES are induced following dsRNA (TLR-3) and LPS (TLR-4) stimulation in a MyD88-independent manner. TLR3 and 4 induce IRF-3 and IRF-7 activation. Here we show that the related transcriptional regulator IRF-5 is specifically induced following TLR-7 and -8 activation by resiquimod, but not following stimulation of TLR-3 by dsRNA. In contrast to the specific induction of IRF5 by TLR-7/8, TLR-3, -4, -7 and -8 all activate IRF-3 and IRF-7. Studies with primary macrophages and dendritic cells show that TLR-7 signaling to type 1 IFN and RANTES is completely abrogated in macrophages or dendritic cells from MyD88-deficient mice while TLR-3/4 signaling is intact. Furthermore we show that the TIR domain containing adapter molecule TRIF, is essential for the TLR-7/8 signaling pathway to IRFs. Studies with dominant negative mutants also establish that the non-canonical IκB kinases, IKKε and TBK1 mediate the IRF-3, -5 and -7 activation pathway downstream of TLR-7/8. Taken together these observations suggest that TLR-7/8 like TLR-3 and -4 induce IRF target gene expression, however TLR-7 signaling is completely dependent on MyD88.

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AUGMENTATION OF THE ANTI-TUMOUR EFFICACY OF CHEMOTHERAPY USING ERYTHROPOIETIN

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Erythropoietin (Epo) is a glycoprotein hormone stimulator of erythropoiesis, administered to cancer patients to treat chemotherapy-induced or tumour-induced anaemia. Hypoxia occurs in areas of tumours with an inefficient vascular supply, leading to chemotherapeutic resistance. Given that Epo may function to increase oxygen delivery to tumour tissue, we investigated the effect of Epo in combination with chemotherapy in a murine model of Lewis Lung Carcinoma (LLC).
In an *in vitro* study, the anti-proliferative effects of the chemotherapy drugs, cisplatin and gemcitabine, were assessed under both hypoxic and normoxic growth conditions. In an *in vivo* study, LLC tumour-bearing mice were randomised into groups receiving chemotherapy (cisplatin and gemcitabine) or chemotherapy combined with a once weekly dose of Epo (darbepoetin alfa). Blood haemoglobin concentration, tumour volume and other biological tumour endpoints were measured.
Tumour cells were more sensitive to the anti-proliferative effects of chemotherapies under normoxic growth conditions compared to hypoxic conditions. *In vivo*, chemotherapy-induced anaemia was corrected using Epo. Epo combined with chemotherapy significantly decreased tumour growth relative to chemotherapy alone.
In conclusion, Epo combined with chemotherapy in our model attenuates tumour growth more effectively than chemotherapy alone. This may be due to increased oxygenation within tumours of Epo-treated animals.

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HuCAL® - HUMAN ANTIBODIES IN THE DEVELOPMENT OF THERAPEUTICS AGAINST CYTOKINE TARGETS

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MorphoSys' Human Combinatorial Antibody Library (HuCAL®) has been proven to be a rich source of high-quality human antibodies selected against antigens as different as haptens, peptides, proteins or cell surface bound molecules.
Antibodies designed to target cytokines *in vivo* must exhibit especially high affinities due to the soluble character of the antigen and the need to compete with the high affinity binding of cytokines to their receptors. The modular structure of the HuCAL® library allows rapid affinity optimisation (as well as fast conversion into different antibody formats) making it extremely suitable for the generation of antibodies with subnanomolar binding properties. Various *in vitro* screening assays for testing the functional quality of such antibodies have also been established at MorphoSys.
Additionally, in a partnership with Oridis Biomed MorphoSys has preferred access to one of Europe's largest high quality tissue banks, containing more than 1.4 million diseased and non-diseased tissues.
In own and partnered programs we generated therapeutic antibodies against inflammatory diseases and for oncological applications. Antibodies were generated that exhibit subnanomolar affinity and potent tumoricidal activity. Examples are given that show efficacy *in vitro* and *in vivo*.

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SYNERGISTIC ACTIVATION OF TLR SIGNALING BY DOUBLE-STRANDED RNA AND CPG DNA MEDIATED BY PKR AND INTERFERON-DEPENDENT PATHWAYS

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Double-stranded RNA (dsRNA), a pathogen associated molecular pattern (PAMP) of viruses and CpG DNA a PAMP associated with bacteria, signal through Toll-like receptor 3 (TLR3) and TLR9, respectively. Co-transfection of human TLR3 and TLR9 into 293T cells supported synergistic activation of an IL-8 promoter reporter construct by poly I:C (pIC), a dsRNA analog. dsRNA and CpG DNA combine to provide enhanced synergistic stimulation of murine macrophages as measured by nitric oxide (NO) and IL-12p40 production. Neutralizing antibodies for IFN-□eta indicate that synergy is mediated in part by paracrine/autocrine effects of IFN-□ in combination with mechanisms independent of IFN-□. PKR is also implicated in the pathway through interaction with TLR signalling components and MKK6. Synergy could also be detected at the level of gene activation with a unique transcription profile induced by the combination. Thus, the combined detection of these PAMPs may represent a condition of definitive pathogen recognition that directs an enhanced, synergistic immune response mediated in part by IFN.

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CURE OF INTESTINAL INFLAMMATION BY CD4+ CD25+ REGULATORY T CELLS

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$CD4^+CD25^+$ T_R cells have been shown to prevent T cell mediated immune pathology in a number of model systems. Here, we show that T_R cells also cure established intestinal inflammation in the T cell transfer model of inflammatory bowel disease. Resolution of colitis is accompanied by proliferation and accumulation of T_R cells locally in the colon and in the mesenteric lymph nodes where they act to inhibit the proliferation of pathogenic T cells. Strikingly, proliferating T_R cells are positioned between clusters of pathogenic T cells and DC suggesting that some of the suppressive activities of T_R cells in vivo may be mediated via effects on DC. We also show that T_R cells can inhibit bacterially triggered T cell independent intestinal inflammation. In this system T_R cells act to prevent sustained activation of the innate immune response via mechanisms involving IL-10 and TGF-beta. Taken together these data illustrate the key role that T_R cells play in negatively regulating immune pathology mediated by both innate and adaptive immune mechanisms and suggest T_R cells may be useful in the treatment of inflammatory diseases.

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IL-10-SECRETING REGULATORY T CELLS – THEIR INDUCTION AND ROLE IN INFECTION

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$CD4^+$ T regulatory (Tr) cells play a critical role in the maintenance of tolerance and in the prevention of autoimmune diseases, but also appear to play a role in immunity to infection. We have established IL-10-secreting Tr1 clones from patients chronically infected with hepatitis C virus and from the lungs of mice infected with *Bordetella pertussis*. Experiments with naïve T cells from TCR transgenic mice demonstrated that the induction of Tr1 cells was directed by DC modulated with pathogen molecules that promote IL-10 and inhibit IL-12 production and suggested that IL-10 was a differentiation factor for Tr1 cells. Experiments in TLR4-defective and IL-10 knockout mice suggested that TLR-4 mediated innate IL-10 production activates antigen-specific Tr1 cells, which conferred resistance to *B. pertussis* by inhibits inflammatory pathology in the lungs. In an attempt to generated Tr cells in naïve mice, we demonstrated that filamentous haemagglutinin from *B. pertussis* and cholera toxin, which enhance IL-12 and synergise with LPS in promoting IL-10 production by DC or macrophages could drive the induction of Tr1 cells in vivo. These molecules and the Tr1 cells that they induce are being assessed for their ability to inhibit Th1-mediated responses in murine models of immune-mediated diseases.

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IL-27, A HETERODIMERIC CYTOKINE COMPOSED OF EBI3 AND P28 PROTEIN, INDUCES PROLIFERATION OF NAIVE $CD4^+$ T CELLS

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An efficient Th1-driven adaptive immune response requires formation of a functional immunological synapse between a naive T cell and an activated antigen-presenting cell, e.g. dendritic cell. Through appropriate activation of the T cell receptor and engagement of co-stimulatory molecules a pathogen encounter is communicated between these cells initiating the adaptive phase of the immune response. In addition, secretion of cytokines, such as the T cell stimulatory cytokine IL-12, by activated antigen-presenting cells is required. IL-12 triggers Th1 polarization of naive $CD4^+$ T cells and secretion of IFN-γ. We describe a new heterodimeric cytokine termed IL-27 that consists of EB13, an IL-12p40-related protein, and p28, a newly discovered IL-12p35-related polypeptide. IL-27 is an early product of activated antigen-presenting cells and drives rapid clonal expansion of naive but not memory $CD4^+$ T cells. It also synergizes with IL-12 to trigger IFN-γ production by naive $CD4^+$ T cells and primary human NK cells. IL-27 mediates its biologic effects through the orphan cytokine receptor WSX-1/TCCR.

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SOCS3- A CENTRAL REGULATOR OF IL-6/GP130 SIGNALING AND INFLAMMATION

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Both SOCS1 and SOCS3 can inhibit JAK tyrosine kinase activity. SOCS1 directly binds to the activation loop of JAKs through the SH2 domain, while SOCS3 SH2 domain binds to the cytokine receptors. SOCS3 SH2 domain has been shown to bind to Y757 of gp130,
We have shown that SOCS3 prevents inflammation by suppressing IL-6/STAT3 signaling in tissues. We also demonstrate that SOCS3 is a key regulator of gp130 in macrophages. In macrophages lacking the SOCS3 gene or carrying a mutation of the SOCS3 binding site (Y759F) in gp130 not only IL-10 but also IL-6 suppressed LPS-induced TNFα production. SOCS3 protein was strongly induced by both IL-6 and IL-10 in the presence of LPS, but selectively inhibited IL-6 signaling. Selective inhibition was due to SOCS3 binding the IL-6 receptor, gp130 but not to the IL-10 receptor. These data indicate that SOCS3 selectively blocks IL-6 signaling, interfering with its ability to inhibit LPS signaling. Consistent with these data, mice specifically lacking the SOCS3 gene in macrophages and neutrophils were resistant to acute inflammation as modelled by LPS-shock. Thus, suppression of SOCS3 in macrophages may represent a novel therapeutic approach for treatment of inflammatory diseases in which IL-6 plays progressive roles.

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EFFECTS OF INTERLEUKIN-23 TREATMENT IN EXPERIMENTAL MURINE CRYPTOCOCCOSIS

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Interleukin (IL)-23 is a recently discovered heterodimeric cytokine consisting of a 40kDa subunit originally described for IL-12 disulfide-bridged with a novel 19kDa protein. Despite of structural similarity to IL-12, however, there is evidence that IL-23 promotes different immunological mechanisms. Thus, our objective was to define the effects of IL-23 vs. IL-12 treatment in a fungal infection model where resistance depends on cell-mediated immunity.
For this purpose we infected C57BL/6 mice with *Cryptococcus neoformans*, an opportunistic fungal pathogen, and supplemented either IL-23, IL-12 or saline solution (PBS). We studied survival and fungal organ burden and analyzed the cytokine profile in serum.
For the observed time period not only IL-12 but also IL-23 therapy prolonged survival significantly compared to the PBS control. However, only IL-12 treatment resulted in complete survival. At day 21 post infection the IL-23 treated mice as well as the IL-12 group had a significantly lower fungal burden in the brain than the control-treated mice. While IL-12 treatment is associated with elevated serum levels of interferon-γ, tumor necrosis factor-α and nitric oxide, the effects observed in the IL-23-treated group appear to based on different mechanisms which remain to be elucidated.

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